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MOLECULAR VARIABILITY OF DIFFERENT SPECIES OF CYATHOSTOMINS IN SELECTED HOSTS

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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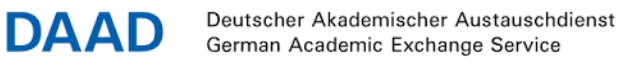


RESUMO

Variabilidade molecular de diferentes espécies de ciatostomíneos em hospedeiros selecionados

Os ciatostomíneos são parasitas nematodes intestinais importantes dos equídeos e incluem mais de 50 espécies reconhecidas como tal. A sua taxonomia tem sido frequentemente revista e a presença de espécies crípticas foi sugerida algumas vezes. Além disso, análises filogenéticas baseadas na morfologia ou sequências moleculares dão, por norma, resultados divergentes. Neste estudo, as sequências nucleotídicas do segundo espaçador interno transcrito (ITS-2) e citocromo c oxidase I (COI) do DNA ribossómico nuclear e do DNA mitocondrial, respetivamente, foram determinadas para adultos de seis espécies de ciatostomíneos (*Cylicocyclus nassatus*, *Coronocyclus labiatus*, *Coronocyclus coronatus*, *Cylicostephanus longibursatus*, *Cylicostephanus minutus*, *Cylicostephanus calicatus*) de hospedeiros de duas regiões geográficas (cavalos domésticos da Alemanha; um Przewalski, um burro, um kulan, uma zebra e um cavalo da Ucrânia). As árvores de máxima verossimilhança foram calculadas para conjuntos de dados de ITS-2, COI e combinados. Não foi detetada nenhuma diferenciação óbvia entre regiões geográficas, nem entre espécies hospedeiras equinas. O ITS-2 foi incapaz de separar *Coronocyclus coronatus* e algumas sequências de *Cylicostephanus calicatus*. Embora as sequências de COI as distinguissem facilmente, revelaram também uma estreita relação entre essas duas espécies. Para *Cylicostephanus minutus* e *Cylicostephanus calicatus* foram detetadas espécies crípticas. *Cylicocyclus nassatus* e *Coronocyclus labiatus* apresentaram diversos haplótipos mitocondriais e nucleares, enquanto que *Cylicostephanus longibursatus* foi comparativamente mais homogéneo. Em conclusão, a análise combinada de haplótipos nucleares e mitocondriais das mesmas espécies, melhorou a resolução das análises e deve ser aplicada a mais espécies e hospedeiros de várias regiões geográficas.

Palavras-chave: Strongylidae, Cyathostominae, Equídeos, análise filogenética, ITS-2, COI.

ABSTRACT

Molecular variability of different species of cyathostomins in selected hosts

Cyathostomins are important intestinal nematode parasites of equids and include more than 50 accepted species. Their taxonomy has been frequently revised and sometimes the presence of cryptic species was suggested. Besides, usually molecular- and morphology-based phylogenetic analyses give divergent results. In this study, the nucleotide sequences of the second internal transcribed spacer (ITS-2) and cytochrome c oxidase subunit I (COI) of nuclear ribosomal DNA and mitochondrial DNA, respectively, were determined for adults of six cyathostomin species (*Cylicocyclus nassatus*, *Coronocyclus labiatus*, *Coronocyclus coronatus*, *Cylicostephanus longibursatus*, *Cylicostephanus minutus*, *Cylicostephanus calicatus*) from hosts of two geographic regions (domestic horses from Germany; a Przewalski horse, a donkey, a kulan, a zebra and a horse from Ukraine). Maximum likelihood trees were calculated for ITS-2, COI and combined data sets. No obvious differentiation was detected between geographic regions, nor equine host species. The ITS-2 was unable to separate between *Coronocyclus coronatus* and some *Cylicostephanus calicatus* sequences. Although COI sequence-based analysis easily distinguished them, they also revealed a close relationship between these two species. Cryptic species were detected in *Cylicostephanus minutus* and *Cylicostephanus calicatus*. *Cylicocyclus nassatus* and *Coronocyclus labiatus* showed diverse and mixed mitochondrial and nuclear haplotypes, while *Cylicostephanus longibursatus* was comparatively homogenous. In conclusion, combined analysis of nuclear and mitochondrial haplotypes from the same specimen, improved resolution of analyses and should be applied to more species and hosts from various geographic regions.

Key words: Strongylidae, Cyathostominae, Equids, phylogenetic analysis, ITS-2, COI.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND SYMBOLS

BIC – Bayesian Information Criterion
bp – base pairs
COI – cytochrome c oxidase subunit I
Cor. – Coronocyclus
Cya. – *Cyathostomum*
Cyc. – Cylicocyclus
Cy-GALA-1 – cyathostomin gut-associated larval antigen-1
Cys. – Cylicostephanus
E. – Escherichia
EL3 – early third-stage larvae
EPG – eggs per gram
ESI – electrospray ionization
ETS – external transcriber spacer
FEC – faecal egg count
Ig – Immunoglobulins
IGS – intergenic non-transcriber spacer
ITS – internal transcribed spacers
ITS-1 – first internal transcribed spacer
ITS-2 – second internal transcribed spacer
L1 – first-stage larvae
L2 – second-stage larvae
L3 – third-stage larvae
L4 – fourth-stage larvae
L5 – pre-adult
LB – Lysogeny Broth
LL3 – late third-stage larvae
MALDI – matrix-assisted laser desorption ionization
MS – mass spectrometry
MS/MS – tandem mass spectrometry
mtDNA - mitochondrial DNA
NAD4 – NADH dehydrogenase subunit 4
OTU – Operational taxonomical unit
PCR – polymerase chain reaction
rDNA – ribosomal DNA
rRNA – ribosomal RNA
S. – Strongylus

SH – Shimodaira-Hasegawa

TAE – TRIS-Acetate-EDTA

ToF – time of flight

UV – ultraviolet

X-gal – 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1: INTRODUCTORY NOTES

1.1 TRAINEESHIP AT THE FREIE UNIVERSITÄT BERLIN

The present master thesis is based on an internship at the Institute of Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin, in Berlin, Germany. It started at 1st of February 2018 until to 2nd of May 2018, under the supervision of Prof. Doctor Georg von Samson Himmelstjerna and co-supervision of Prof. Doctor Luís Madeira de Carvalho, in compliance with the requirements for the acquisition of the Integrated Master's in Veterinary Medicine degree by the Faculty of Veterinary Medicine, University of Lisbon.

Through the internship, I was integrated in the PhD project of Christina Bredtmann about "Comprehensive molecular and proteomic cyathostomin species delineation", where I learned about molecular biological techniques that allowed me to work independently. There, I worked with cyathostomin DNA samples from which I did several polymerase chain reactions (PCRs), PCR product purification and molecular cloning. Besides this, I also learned how to analyze the sequences obtained with appropriate software such as Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7®) and had an introduction to phylogenetic analysis.

During my time there, I had the opportunity to meet and work with incredible persons who taught me about their own projects, the importance of teamwork and I had the chance to participate in the 28th Annual Meeting of the German Society for Parasitology by helping in the organization.

1.2 THE RESEARCH PROJECT

This master thesis is based on a part of the PhD project of Christina Bredtmann, about "Comprehensive molecular and proteomic cyathostomin species delineation". During this project a first protocol on concurrent proteomic profiling and molecular characterization of individual cyathostomin worms was developed. It included a collaboration with Dra. Tetiana Kuzmina, from the I.I. Schmalhausen Institute of Zoology at the National Academy of Sciences of Ukraine, one of the very few experts in the world who can identify adult cyathostomins morphologically to the species level. Christina Bredtmann and Dr. Tetiana Kuzmina analyzed some of the samples.

With their permission, I use in this thesis the information and data collected by them and complemented by the results I obtained through my three months of internship. Apart from this, I did all the phylogenetic and statistical analysis presented in this thesis, with the guidance of Doctor Jürgen Krücken.

CHAPTER 2: INTRODUCTION

Worldwide, equids are exposed to intestinal helminth infections that can compromise their health and welfare. The biodiversity of parasitic nematode species in horses is ample but the great majority belongs to the family Strongylidae. The species from this family are divided in the subfamilies Strongylinae (strongylins) and Cyathostominae (cyathostomins), which morphologically differ, among other criteria, by the form of the buccal capsule and to some extend in their respective size (Lichtenfels, Kharchenko, & Dvojnos, 2008). Although Strongylidae comprise the most pathogenic nematodes of horses, information on these nematodes is to date mainly focused on characteristics of their morphology, life cycle, prevalence and disease control and prevention (Lyons, Kuzmina, Tolliver, & Collins, 2011; Morariu et al., 2016) and particularly limited concerning the genetic and molecular characteristics of cyathostomins.

Cyathostomins are currently considered the most important horse nematodes (Lyons, Tolliver, & Drudge, 1999), especially due to the reduced prevalence of *Strongylus* spp. and spread of cyathostomin populations showing anthelmintic resistance (Traversa et al., 2010). They are ubiquitous and inhabit the large intestine of infected equids with high prevalence and abundance. Most equids can harbor thousands of cyathostomins without developing clinical disease, but in some cases, it can lead to a clinical syndrome called “larval cyathostominosis” with sometimes 50% fatality rate (Giles, Urquhart, & Longstaffe, 1985; Eysker, Boersema, & Kooyman, 1989, 1990; Love & Mckeand, 1997; Love, Murphy, & Mellor, 1999; Traversa et al., 2010). This group contains 50 species (Lichtenfels et al., 2008). However, little is known of the ecology of the individual species or how they interact with one another in the host or in external environment. The accurate identification of cyathostomins is crucial in order to study their biology, epidemiology and pathogenicity. Most data are obtained through post-mortem examinations or after anthelmintic treatments, often complicated by the difficulties in accurate microscopic identification of adult specimens, which require an expert morphologist. Additionally, the eggs of these subfamilies are indistinguishable to genus/species level and only sometimes third stage larvae can be identified to the genus level (Traversa et al., 2010).

For these reasons, different molecular techniques have been developed not only to overcome the limitations of morphological identification, but also to further clarify the taxonomy and evolutionary relationships between cyathostomins, as well to improve the control of parasite populations and preserve the effectiveness of anthelmintics.

CHAPTER 3: LITERATURE REVIEW

The aim of this review is to make the reader familiar with the importance of parasitic nematodes of equids focusing on the actual knowledge regarding the members of the family Strongylidae, including the differences and similarities between the two subfamilies (Strongylinae and Cyathostominae). In addition, it's presented the technological and molecular advances that have been achieved to better understand and control these species, while at the same time, preserving the effectiveness of anthelmintic compounds for as long as possible.

3.1 IMPORTANCE OF STRONGYLID NEMATODES OF HORSES

A parasite is an organism that has lost its physiological independence and relies on another organism (the host), for example to acquire its nutritional requirements and other metabolic needs, on the cost of and associated with damaging the host. This physiological association is called parasitism (Watson, 1965). Medical parasitology studies this association in three major groups of animals: parasitic protozoa, helminths and arthropods, that directly cause disease or act as a vector of various pathogens (Castro GA, 1996b). Helminths, commonly called "worms", are invertebrates characterized by elongated, flat or round bodies and they do affect not only animals, but also humans and plants. They are separated according to their general external shape, the host organ they inhabit, and the definitive classification is based on the external and internal morphology of egg, larval and adult stages (Castro GA, 1996a). The helminths of veterinary importance are divided in two major phyla, Nematoda (roundworms) and Playhelminthes (flatworms), and a minor phylum, Acanthocephala. Trematoda (flukes) and Cestoda (tapeworms) are the parasitic groups in the phylum Platyhelminthes (Taylor, Coop, & Wall, 2016b). Nematoda is an ancient and biologically diverse phylum, and its species diversity may be 1 million or more (Blaxter & Koutsovoulos, 2015). The great majority are free-living, and only some smaller portion exploit animals for part of their life cycle. Parasitism of animals or plants has evolved at least 15 times independently in the Nematoda (Blaxter & Koutsovoulos, 2015).

Equids worldwide are the hosts of several gastrointestinal nematodes and are exposed through grazing on contaminated pastures. If not treated with effective anthelmintics, they can accumulate large numbers of worms in individual host, compromising their health (Matthews, 2014). There are 83 species of nematode parasites of equids, belonging to 29 genera, 12 families and 7 suborders. From these, 64 species belong to the Strongylidae family (Lichtenfels et al., 2008).

3.2 FAMILY STRONGYLIDAE

Worldwide, the great majority of nematode parasites of equids are members of the family Strongylidae, which includes the most common and pathogenic nematode parasites of horses. The Strongylidae (common name strongylids) of horses are nematodes with a well-developed buccal capsule, a mouth collar with two leaf-crowns, and in males a strongylid copulatory bursa. The family can be separated into two subfamilies: Strongylinae (common name strongylins), usually large or medium-sized with a large globular or funnel-shaped buccal capsule; and Cyathostominae (common name cyathostomins), usually small to medium-sized with a small cylindrical buccal capsule (Lichtenfels et al., 2008).

Through many years, strongylins (particularly *Strongylus vulgaris*) were considered the main pathogens of domestic equids, and veterinarians tended to regard cyathostomins as having low or limited pathogenicity. However, since the end of the last century, cyathostomins are considered the principle parasitic pathogen of the horse (Love et al., 1999). This rise coincides with the marked decrease in prevalence of *Strongylus vulgaris* infections and the widespread appearance of anthelmintic resistance in cyathostomins (Love et al., 1999; Kaplan, 2002; Von Samson-Himmelstjerna, 2012).

Strongylids of horses all have direct life cycles, with no intermediate hosts (Nielsen & Reinemeyer, 2018a), and their free-living stages are capable of survival in wide extremes of weather and climate, with third-stage larvae (L3) surviving temperatures from -8ºC to 38ºC (Nielsen, Kaplan, Thamsborg, Monrad, & Olsen, 2007). The eggs of both subfamilies, Strongylinae and Cyathostominae, cannot be differentiated microscopically, and the only practical method of differentiation (other than molecular approaches) is morphological characterization of the third larval stages obtained through coproculture (Nielsen & Reinemeyer, 2018a).

3.2.1 SUBFAMILY STRONGYLINAE

The Strongylinae of domestic equids are organized in 5 genera: *Strongylus*, *Oesophagodontus*, *Triodontophorus*, *Bidentostomum* and *Craterostomum*, with a total of 14 species (Lichtenfels et al., 2008). Some strongylins prefer one or more specific host species and they are distributed worldwide or restrained to a specific geographical area (Table 1). The term “large strongyles” is often used to describe this subfamily or only the genus *Strongylus*. Additionally, “small strongyles” is being used to describe the Cyathostominae or all the strongylid species except for the genus *Strongylus* (Love et al., 1999; Lyons, Tolliver, & Drudge, 1999; Bu, Niu, & Zhang, 2013). To avoid confusion, the term “large” and “small strongyles” will not be used in this thesis.

The species belonging to the genus *Strongylus,* especially *Strongylus vulgaris*, are considered the most pathogenic nematodes of equids (Lyons et al., 1999; Nielsen & Reinemeyer, 2018a). While they are now considered less important than cyathostomins, due to their lower prevalence and susceptibility of anthelmintic treatments, strongylins still are a major concern in horse parasite control (Mittel et al., 2012).

The species belonging to the genera *Oesophadodontus, Craterostomum, Bidentostomum* and *Triodontophorus* are less common than the *Strongylus* species, and the currently sparse information regarding their individual life cycle and pathogenicity describe them as being similarly relevant to cyathostomins, despite the morphological and taxonomical differences (Taylor et al., 2016b).

Table 1 - Genus and species belonging to Strongylinae subfamily, equid host species and distribution (based on Lichtenfels et al., 2008).

			Hosts (Equus)							
	Genus	*Species*	*E. caballus*	*E. caballus x E. asinus*	*E. asinus*	*E. przewalskii*	*E. hemionus*	*E. quagga burchellii*	*E. hartmannae*	*E. zebra*	*Distribution*
STRONGYLINAE	*Bidentostomum*	*B. ivaschkini*	x	x							Asia
	Craterostomum	*C. acuticaudatum*	x	x	x	x	x	x	x		Cosmopolitan
	Oesophagodontus	*O. robustus*	x	x	x	x		x			Cosmopolitan
	Strongylus	*S. asini*			x			x	x		Africa, Asia, North America
		S. edentatus	x	x	x	x	x	x			Cosmopolitan
		S. equinus	x	x	x	x	x	x			Cosmopolitan
		S. vulgaris	x	x	x	x	x	x			Cosmopolitan
	Triodontophorus	*T. brevicauda*	x	x	x	x	x	x			Cosmopolitan
		T. burchelli						x			Africa
		T. hartmannae							x		Africa
		T. minor			x		x				Europe, Asia, Africa
		T. nipponicus	x			x	x				Asia, Europe, North and South America
		T. serratus	x	x	x	x	x	x			Cosmopolitan
		T. tenuicollis	x	x	x	x	x	x			Cosmopolitan

3.2.1.1 LIFE CYCLE

Although the adult members of this subfamily live in the large intestine of equids, they have differences in their life cycle (Figure 1). Based in their life cycle, the strongylins can be divided in two groups: migratory, with the 4 species from the genus *Strongylus*; and non-migratory, the other 10 species (Taylor et al., 2016b). Nevertheless, for all the strongylins, the life cycle has a common path: the fertilized eggs are shed by adult females in the large intestine, and excreted to the environment in the feces, where they embryonate before first-stage larvae (L1) hatch. Under optimal temperature (25–33ºC) and humidity (57–63%) conditions (Lyons et al., 1999), the L1 develop into second-stage larvae (L2) in the feces and then into third-stage larvae (L3), the infective stage. The L3 leaves the feces and migrates to the forage, where it is ingested by a horse. After ingestion, the parasitic larval development will differ depending on the specie: for non-migratory species, such as *Triodontophorus* spp., the L3 invade the mucosa of the large intestine, encyst, undergo some stages of development, including in particular molting to fourth-stage larvae (L4), and eventually re-emerge to molt to pre-adults (L5) and further development to adults. The prepatent period is thought to be approximately 2-3 months (Taylor et al., 2016b; Nielsen & Reinemeyer, 2018a). For migratory species of the genus *Strongylus*, each specie has a different migration path:

Strongylus edentatus: L3 cross the intestinal mucosa and travel via the portal system reaching the liver parenchyma where they molt to L4 and continue migrating through the liver, with larvae found around the hepatorenal ligament. Then, they travel under the peritoneum to many sites, with a predilection for the flanks and hepatic ligaments. After 4 months the larvae molt to pre-adults and migrate under the peritoneum to the wall of the large intestine where a large purulent nodule is formed, which subsequently ruptures with release of the young adult parasite into de lumen. The prepatent period is 9-10 months (McCraw & Slocombe, 1978; Taylor et al., 2016b).

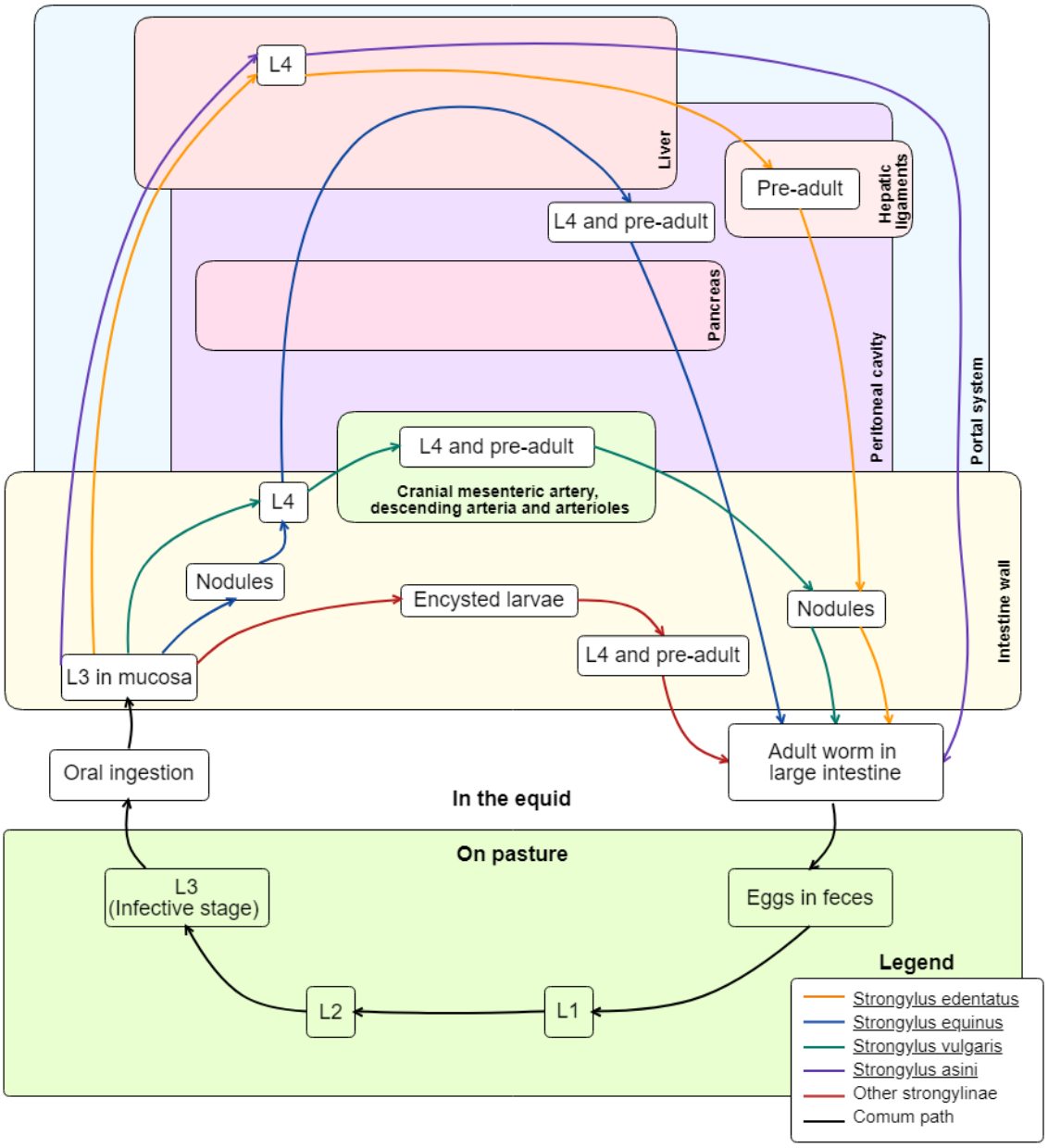
Strongylus equinus: L3 penetrate the wall of the large intestine provoking nodules in the muscular and subserosal layers. There, molts occur before they migrate across the peritoneal cavity and through the pancreas to reach the liver, where L4 persist for several weeks. Later, L4 and pre-adult larvae migrate to the pancreas and peritoneal cavity, where they can be found before appearing in the large intestinal lumen as adults. The prepatent period is 8-9 months (McCraw & Slocombe, 1985; Nielsen & Reinemeyer, 2018a).

Strongylus vulgaris: L3 penetrate the intestinal mucosa and molt to L4 in the submucosa within a few days. Then, they enter small arteries of the intestine and there is a progressive migration up the arterial tree until they reach the cranial mesenteric artery and its descending arteria and arterioles, where L4 stay for 3-4 months. The larvae molt to pre-adults and migrate down the arteries to the intestinal wall, where nodules are formed on the intestinal serosa surface. Due

to their size, the larvae can travel no further in the arteries and subsequent rupture of these nodules releases young adults into the intestinal lumen. The prepatent period is approximately 6 months (Duncan, 1973; McCraw & Slocombe, 1976; Taylor et al., 2016b).

Strongylus asini: Migration and development of *S. asini* in the host are not completely known. L3 cross the intestinal mucosa and travel via the portal system reaching the liver parenchyma forming nodules where they molt to L4. Then, parasites return via the portal system until reaching the large intestinal lumen as young adults (Malan, De Vos, Reinecke, & Pletcher, 1982; Nielsen & Reinemeyer, 2018a).

Figure 1 - Life cycle of *Strongylus* species and other strongylins (original; based on Duncan, 1973; McCraw & Slocombe, 1976, 1978; Malan et al., 1982; McCraw & Slocombe,1985; Nielsen & Reinemeyer, 2018a; Taylor et al., 2016b).



3.2.1.2 PATHOGENICITY AND CLINICAL SIGNS

In the adult stage, strongylins are found attached to the large intestinal mucosa. Their large buccal capsules enable them to remove plugs of mucosa and to ingest nutrients in the form of blood, plasma or mucosal cells. They may cause focal inflammation and ulceration (Nielsen & Reinemeyer, 2018d). Anemia, emaciation, poor coat and poor performance are frequently attributed to the presence of strongylins in the intestine (McCraw & Slocombe, 1976). Since strongylins are rarely present in high numbers, the major pathology is caused by their migratory larval stages (Nielsen & Reinemeyer, 2018d).

For the migratory strongylins, the pathology varies within the species, since each has a different migratory path. Migratory *S. edentatus* larvae has been associated with liver pathology and peritonitis (McCraw & Slocombe, 1978; Nielsen & Reinemeyer, 2018d), being prone to cause parenchymal scars of fibrous tissue in the liver, subperitoneal hematomas, hemorrhage, peritonitis and omental adhesions (Taylor, Coop, & Wall, 2016a). *S. equinus* larvae, like *S. edentatus*, migrate through the liver, causing similar pathology (Taylor et al., 2016a). But in addition, it passes through the pancreas, favoring the appearance of pancreatic lesions, including pancreatitis with subsequent pancreatic dysfunction (McCraw & Slocombe, 1985; Nielsen & Reinemeyer, 2018d). For *S. asini*, very little information is available but it causes numerous nodules in the liver (Malan et al., 1982), and peritonitis has also been associated with this infection (Colglazier & Jaskoski, 1956).

Even being now less common than it was 30 years ago, *S. vulgaris* is still the most significant and pathogenic nematode parasite of horses (Lyons et al., 1999; Taylor et al., 2016a). The migration of the *S. vulgaris* larvae through the cranial mesenteric artery and its main branches damages the endothelium, causing endoarteritis with marked inflammation and thickening of the arterial wall (McCraw & Slocombe, 1976; Taylor et al., 2016a). This leads to the formation of thrombi causing thromboembolism which may result in colic and intermittent lameness, if it affects the external iliac artery (McCraw & Slocombe, 1976). Although uncommon, true aneurysms with dilation and thinning of the arterial wall may also occur, especially in animals with repeated infections (Taylor et al., 2016a). The severity of the signs is related to the number of larvae ingested and to the age and previous experience of the host (McCraw & Slocombe, 1976).

Concerning clinical signs, both *S. edentatus* and *S. equinus* may cause diarrhea, fever, edema, anorexia, depression and weight loss. *Strongylus vulgaris* may cause anemia, poor condition and performance, varying degrees of colic, lameness, intestinal stasis, intestinal rupture and death (Taylor et al., 2016a).

Regarding the pathogenicity of the non-migratory strongylins, little information is available. Members of the genus *Triodontophorus* are found in large numbers in the colon and contribute to the deleterious effects of mixed strongyle infection by damaging the mucosa through the adults' feeding habits. They may cause loss of condition, anemia, weakness and diarrhea (Taylor et al., 2016a). The remaining strongylins, may also contribute to pathogenicity of mixed strongyle infection.

3.2.2 SUBFAMILY CYATHOSTOMINAE

Being considered the principal and most prevalent parasitic nematodes of equids, cyathostomins have been target of numerous scientific researches. Virtually, 100% of the horses are infected with cyathostomins (Lyons et al., 1999). The accurate identification of cyathostomins is crucial to study their biology, epidemiology and pathogenicity. However, correct microscopic identification of adult stages requires an expert morphologist to distinguish between at least 50 species of cyathostomins, distributed among 14 genera (Lichtenfels, Gibbons, & Krecek, 2002; Lichtenfels et al., 2008).

Most cyathostomins infect several host species and are globally distributed, but others seem to show preference for specific hosts or for specific geographical regions (Table 2). Despite the large number of species, only a few are dominant (Anjos & Rodrigues, 2003; Lichtenfels et al., 2008; Traversa et al., 2010; Morariu et al., 2016). In a broad scale study from 2010, Traversa et al. found no mono-specific infections in the 102 yards analized across Italy, United Kingdom and Germany, and the range of species simultaneously detected in a single yard was 3 to 13 using the reverse line blot approach, which is not able to identify all species since probes are only available for a subset of 13 very common species.

Also based in feces, but using expelled strongyles after deworming, it was found that the number of species per horse can range between 1 and 17 species of strongylids (Luis M. Madeira de Carvalho et al., 2014). In another recent study, Morariu et al. (2016) found that all the horses studied were infected by 8 to 24 species using parasitological necropsies and morphological differentiation of adult worms.

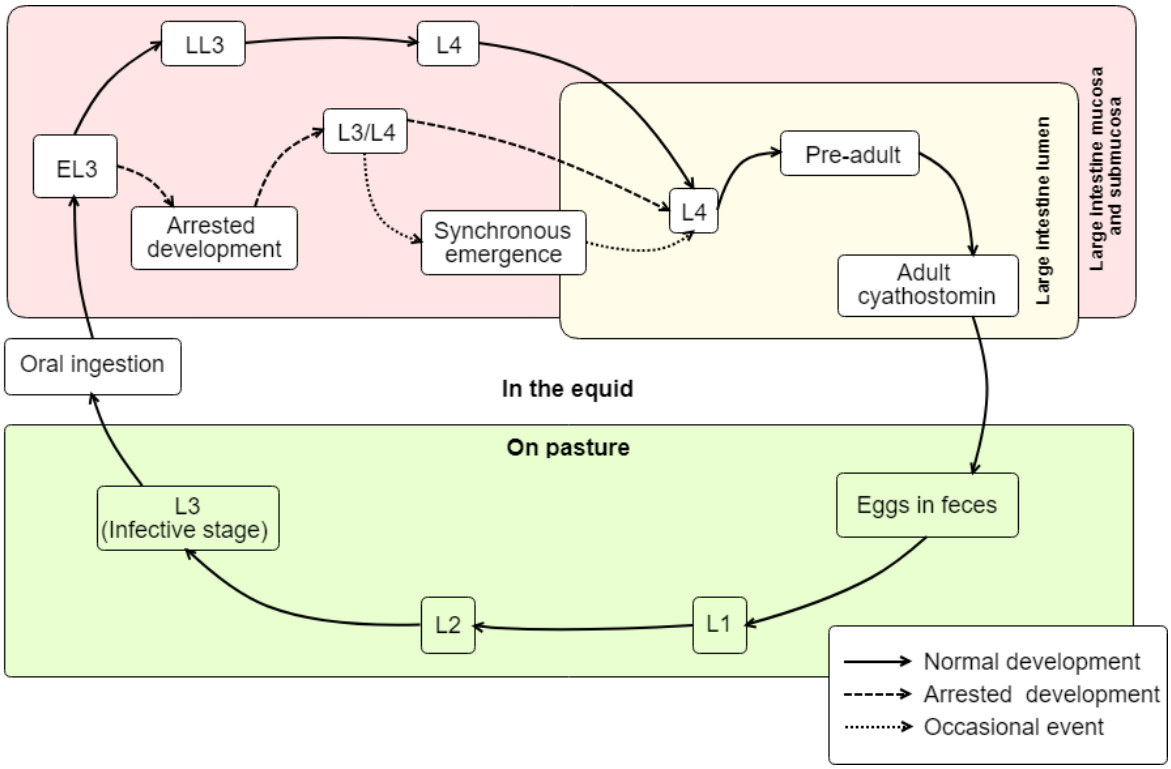
Table 2 - Genus and species belonging to Cyathostominae subfamily, equid host species and distribution (based on Lichtenfels et al., 2008)

	Genus	Species	Hosts (Equus)								Distribution
			E. caballus	*E. caballus x E. asinus*	*E. asinus*	*E. przewalskii*	*E. hemionus*	*E. quagga burchellii*	*E. hartmannae*	*E. zebra*	
CYATHOSTOMINAE	*Caballonema*	*C. longicapsulatum*	x								Asia
	Coronocyclus	*C. coronatus*	x	x	x	x	x				Cosmopolitan
		C. labiatus	x	x	x	x	x	x			Cosmopolitan
		C. labratus	x	x	x	x	x				Cosmopolitan
		C. sagittatus	x	x		x					Europe, Asia
		C. ulambajari	x								Asia
	Cyathostomum	*C. alveatum*	x	x	x			x			Cosmopolitan
		C. catinatum	x	x	x	x	x	x			Cosmopolitan
		C. montgomeryi	x	x				x			Africa
		C. pateratum	x	x	x	x	x	x			Cosmopolitan
		C. tetracanthum	x		x			x			Cosmopolitan
	Cylicocyclus	*C. adersi*			x			x		x	Africa
		C. ashworthi	x	x	x	x	x				Cosmopolitan
		C. asini			x			x		x	Cosmopolitan
		C. auriculatus	x		x			x		x	Asia, Africa
		C. brevicapsulatus	x		x						Asia, Europe, America
		C. elongatus	x	x	x	x	x	x			Cosmopolitan
		C. gyalocephaloides						x			Africa
		C. insigne	x	x	x	x	x				Cosmopolitan
		C. leptostomum	x	x	x	x	x	x			Cosmopolitan
		C. nassatus	x	x	x	x	x	x			Cosmopolitan
		C. radiatus	x	x	x	x	x	x			Cosmopolitan
		C. triramosus						x	x		Africa
		C. ultrajectinus	x		x		x	x			Cosmopolitan
	Cylicodontophorus	*C. bicoronatus*	x	x	x	x	x				Cosmopolitan
		C. reineckei						x	x		Africa
	Cylicostephanus	*C. asymetricus*	x		x						Cosmopolitan
		C. bidentatus	x	x	x	x	x	x			Cosmopolitan
		C. calicatus	x	x	x	x	x				Cosmopolitan
		C. goldi	x	x	x	x	x	x			Cosmopolitan
		C. hybridus	x		x	x					Asia, Europe, North America
		C. longibursatus	x	x	x	x	x	x			Cosmopolitan
		C. minutus	x	x	x	x	x				Cosmopolitan
	Cylindropharynx	*C. brevicauda*		x	x			x			Africa
		C. intermedia						x	x		Africa
		C. longicauda		x	x			x	x		Africa
	Gyalocephalus	*G. capitatus*	x	x	x	x	x	x			Cosmopolitan
	Hsiungia	*H. pekingensis*	x		x						Asia
	Parapoteriostomum	*P. euproctus*	x	x	x	x	x	x			Cosmopolitan
		P. mettami	x	x	x	x	x				Cosmopolitan
		P. mongolica	x								Asia
		P. shuermanni						x			Africa
	Petrovinema	*P. poculatum*	x	x	x	x	x	x			Cosmopolitan
		P. skrjabini	x		x						Asia
	Poteriostomum	*P. imparidentatum*	x	x	x	x	x	x			Cosmopolitan
		P. ratzii	x	x	x	x	x	x			Cosmopolitan
	Skrjabinodentus	*S. caragandicus*	x	x							Asia
		S. longiconus						x	x		South Africa
		S. tshoijoi	x								Asia
	Tridentoinfundibulum	*T. gobi*	x	x							Asia, Europe, North America

3.2.2.1 LIFE CYCLE

Cyathostomins have a direct life cycle and this is very similar for all species (Figure 2). The adults are usually less than 2.5 cm in length and are found in the large intestine, i.e. the cecum and colon of equids. The free-living stages of cyathostomins are similar to those of the strongylins. The fertilized eggs are excreted to the environment in the feces. Under optimal conditions the eggs embryonate, hatch and develop from L1 to L3 within 4 days (Nielsen et al., 2007). Once they reach the L3 (infective) stage, the larvae migrate to the surrounding forage and are ingested by the equids. After ingestion and depending on the species, L3 invade the mucosa or submucosa of the cecum and colon as early third-stage larvae (EL3). Subsequently, a fibrous capsule is formed around the EL3, and from this stage forward, this larva starts to be referred as “encysted”. Then, the encysted EL3 can progress steadily to adulthood or, alternatively, arrest their development and persist as hypobiotic EL3s for more than a year or two. With progressive development, the EL3 develop into a late L3 stage (LL3), which is significantly larger. Remaining encysted, the LL3 molts to L4, which grows and eventually ruptures de cyst wall entering the lumen of the large intestine. There, the L4 molts to the pre-adult (or L5) and gradually develops to the adult stage (Nielsen & Reinemeyer, 2018a). The larval development in the mucosa may be inhibited, a process which is called hypobiosis. These hypobiotic or inhibited larvae often accumulate to large numbers and can later emergence in a synchronized manner leading to significant damage of the mucosa. If no hypobiotic phase occurs, a female cyathostomin can begin to lay eggs as soon as 5 weeks after infection (Round, 1969).

Figure 2 - Life cycle of cyathostomins (original; based on Nielsen & Reinemeyer, 2018a).



3.2.2.2 PATHOGENICITY AND CLINICAL SIGNS

Most horses can harbor thousands of cyathostomins without developing clinical disease (Love et al., 1999). In the adult stages some species may attach to the mucosa, but most reside in the paramucosal ingesta, feeding on organic material (Nielsen & Reinemeyer, 2018d). Some cyathostomins exhibit preferences regarding the site as adults (Tolliver, 2000). The pathogenicity of the adult cyathostomins is usually related to the accumulative effects of multiple parasitic stages occupying the host and can be designated as “chronic cyathostominosis” (Love et al., 1999). The symptoms are non-specific, but include weight loss, dull coat, pot-bellied appearance, colic and loose feces (Nielsen & Reinemeyer, 2018d).

The most severe pathology is caused by the larval stages. After ingestion, the mucosal penetration by the EL3 causes focal inflammation and a fibroblastic reaction in the lamina propria (Love et al., 1999). Following mucosal invasion, the cysts forming around the larvae induce a modest inflammation. The cyst wall permits the passage of nutrients from the host to the larvae, while at the same time preventing the passage of certain types of anthelmintics (Nielsen & Reinemeyer, 2018d). The most pathogenic moment in the cyathostomins life cycle, is the emergence of the encysted larvae, called excystment. The emerging L4 are 10 times larger than the EL3, so the mechanical damaged caused to the mucosa is more severe. A host reaction is mounted not only to the mechanical damage, but also to the materials produced by the larvae during their development, which are released during the excystment. If the cyathostomin burden is small, the excystment site may exhibit focal hemorrhage, congestion and edema. However, in larger burdens, these lesions may coalesce leading to massive inflammation of the large intestine, which becomes extremely edematous, thick and hemorrhagic or necrotic, inducing a protein loss enteropathy, due to increased intestinal permeability. The described findings are associated with a syndrome called “larval or type II cyathostominosis” and as clinical signs show profuse diarrhea (causing dehydration), weight loss, hypoproteinemia, ventral edema and various types of equine colic (Love et al., 1999; Peregrine, McEwen, Bienzle, Koch, & Weese, 2006; Morariu et al., 2016; Nielsen & Reinemeyer, 2018d). Neutrophilia and anemia can also be found in the hemogram (Love et al., 1999). The mortality rate for this syndrome can be as high as 50%, even with proper treatment (Giles, Urquhart, & Longstaffe, 1985; Eysker, Boersema, & Kooyman, 1989, 1990; Love & Mckeand, 1997; Love et al., 1999; Traversa et al., 2010).

Even though some cyathostomin species have preference sites regarding the adult stages, it is not known if the larvae also prefer certain gut sections or if the pathogenicity may vary with the cyathostomin species due to the difficulty to identify these larvae.

3.2.3 EPIDEMIOLOGY

There is seasonal fluctuation in mixed strongylid egg counts, but this varies with geographical location (Duncan, 1973; Lyons et al., 1999). In 1973, Duncan found in the United Kingdom that in the winter months the number of eggs being excreted was the lowest, in the spring, the egg counts start to rise and in the summer, they reach their peak. This makes sense, since both egg and larvae are affected by environmental factors, such as temperature and moisture (McCraw & Slocombe, 1976; Nielsen et al., 2007). A recent study showed that cyathostomins can develop to infective larvae on moist straw bedding (Love, Burden, McGirr, Gordon, & Denwood, 2016). Moreover, the horse sex can also be important regarding the strongylid community structure (Sallé, Kornaś, & Basiaga, 2018). Within the genus *Strongylus*, *S. vulgaris* seemed to be the most prevalent species in Poland (Studzińska, Tomczuk, Demkowska-Kutrzepa, & Szczepaniak, 2012). However, due the efficacy and regular use of anthelmintics, the frequency of strongylins has decreased (Schneider, Pfister, Becher, & Scheuerle, 2014; Studzińska et al., 2012). Nowadays, cyathostomins are the most prevalent horse parasitic nematodes and also occur with the highest intensities (Corning, 2009). Even though more than 50 species are considered to be valid, 13 species can account for 98-99% of the total cyathostomin burden worldwide (Ogbourne, 1976; Reinemeyer, Smith, Gabel, & Herd, 1984; Gawor, 1995a; Chapman, French, & Klei, 2002; Kuzmina, Kharchenko, Starovir, & Dvojnos, 2005; Čerňanská et al., 2009): *Coronocyclus* (*Cor.) coronatus, Cor. labiatus, Cor. labratus, Cyathostomum* (*Cya.*) *catinatum, Cya. pateratum, Cylicocyclus* (*Cyc*.) *ashworthi, Cyc. goldi, Cyc. insigne, Cyc. nassatus, Cylicostephanus* (*Cys.*) *calicatus, Cys. longibursatus, Cyc. leptostomum* and *Cys. minutus*. Some cyathostomins species have a preference for different compartments of the large intestine and the intensity is usually higher in the ventral than the dorsal colon and lowest in the cecum (Morariu et al., 2016; Bellaw et al., 2018; Nielsen & Reinemeyer, 2018d).

The horse infection by strongylids is greatly influenced by the horse keeping conditions, accessibility and contamination level of the pasture, and the management of anthelmintic treatments (Kuzmina, 2012).

3.2.4 DIAGNOSTIC OF STRONGYLID INFECTION

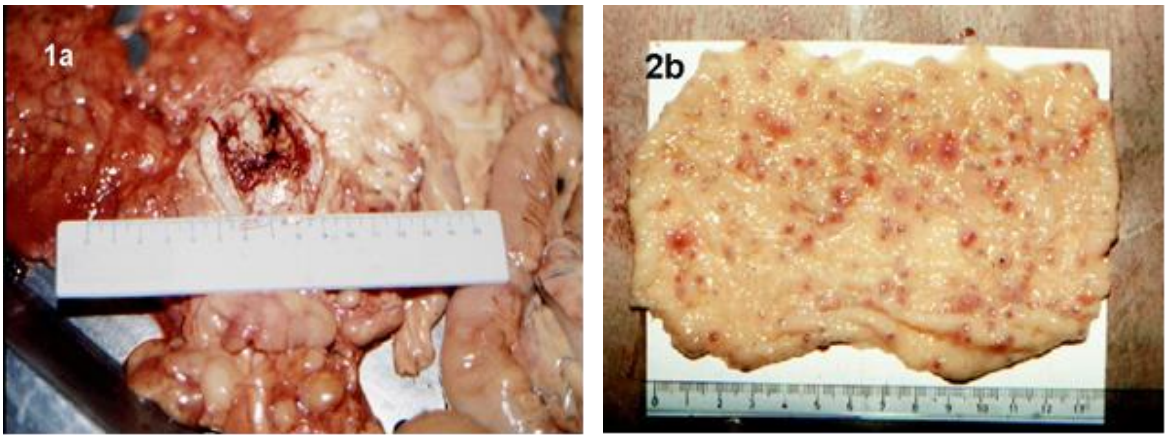
Bearing in mind the clinical signs, the diagnosis can be performed based on the presence of strongylid eggs in qualitative and quantitative fecal analysis techniques, but since the larvae are responsible for the most severe clinical signs, it is possible to have no eggs in fecal analysis when the horses suffer from disease. A typical strongylid egg is about 50 µm × 100 µm, oval-shaped, with a smooth surface and identifiable cells (Nielsen & Reinemeyer, 2018c) (Figure 3). Since the eggs are similar, the distinction between strongylids is usually through L3, obtained using fecal culture techniques (Nielsen & Reinemeyer, 2018c). However, the L3s can

be speciated only for *Strongylus spp*. while cyathostomins can be identified sometimes at best to the genus level (Traversa et al., 2010; reviewed by Bredtmann, Krücken, Murugaiyan, Kuzmina, & von Samson-Himmelstjerna, 2017). Due to the time and experience required for this identification, some molecular, serologic and proteomic techniques are being developed to identify strongylids to the species level (Traversa et al., 2010), which will be discussed further below in this chapter.

Figure 3 – Examples of strongylid eggs (left side) and a *Strongylus vulgaris*' L3 (right side). Source: Madeira de Carvalho, 2002

Ultrasound and rectal palpation can also be used to diagnose *S. vulgaris* infection, by detecting a thickened and dilated cranial mesenteric artery (Greatorex, 1977; Wallace, Selcer, & Becht, 1989). The serum protein concentration is often reduced in cases of larval cyathostominosis (Murphy & Love, 1997). Adults and larvae can be recovered *post-mortem* during a necropsy, which also allows checking for the corresponding lesions of migrating or encysted larval stages (Figure 4).

Figure 4 – Example of a mesenteric artery of a horse with *Strongylus vulgaris* L4 and L5 (left side) and part of an equid cecum with encysted cyathostomin larvae with different sizes and hemorrhagic appearance (right side) during a necropsy. Source: Madeira de Carvalho, 2002



3.2.5 TREATMENT AND MANAGEMENT

The control of equid strongylids can be achieved by blocking any single event from the parasite life cycle. The most common action involves the use of anthelmintics to disrupt life cycle events within the hosts, improving their health and reducing the environmental contamination with eggs and larvae. Currently, there are three major drug classes available for strongylid control in horses: the benzimidazoles, which include fenbendazole and oxibendazole, the tetrahydropyrimidine pyrantel and the macrocyclic lactones, such as ivermectin and moxidectin. The heterocyclic compound piperazine can also be used as anthelmintic but less frequently (Lyons et al., 1999; Corning, 2009; Schneider et al., 2014). Anthelmintics seem to effectively reduce and control the species belonging to the genus *Strongylus* (Studzińska et al., 2012). For cyathostomins, anthelmintic resistance has been reported to all drug classes, being more frequently for benzimidazoles and less often for pyrantel, while macrocyclic lactones were found to still be effective on more than 90% of the farms (Lyons, Tolliver, et al., 2011; Lester et al., 2013; Matthews, 2014; Bellaw et al., 2018). Moxidectin seems to be the most effective anthelmintic against cyathostomins by having high efficacy against all stages (including encysted larvae), and persistent activity against re-infections, allowing less frequent treatment and less selection for resistance (Corning, 2009). Using a rotational program of anthelmintics could, in theory, reduce the number of future resistance cases (Kaplan & Nielsen, 2010).

The selective therapy is a commonly accepted chemical approach by parasitologists only applicable in adult horses. It involves the diagnosis of parasitism and a fecal egg count (FEC) on all horses from the population. The horses that surpass a certain FEC threshold (usually 200 eggs per gram (EPG)) are treated with the anthelmintic specific for the observed parasite (Leathwick & Sauermann, 2018). This approach also creates a *refugia* in the horses by leaving a proportion of untreated parasite population. The maintenance of adequate parasite *refugia* can slow the development of anthelmintic resistance, since they are not selected by treatment and provide a pool of sensitive alleles (Nielsen et al., 2007). However, a publication has illustrated that apparently healthy horses with egg counts below 100 EPG can harbor cyathostomin burdens in the range of 100,000 luminal worms (Nielsen, Baptiste, Tolliver, Collins, & Lyons, 2010), while it can also lead to the reappearance of previously controlled parasites such as *S. vulgaris*, showing that this may not be the best approach regarding strongylids (Nielsen, Vidyashankar, Olsen, Monrad, & Thamsborg, 2012; European Scientific Counsel Companion Animal Parasites (ESCCAP), 2019). The strategic treatment approach is based on the horse's age and usage, applied particularly in foals and young horses which need comprehensive protection with regular anthelmintic treatments (European Scientific Counsel Companion Animal Parasites (ESCCAP), 2019).

Alternative approaches to control and reduce the parasite population to an acceptable level can also be applied. Among them are genetic selection of horses which exhibit enhanced resilience and resistance to parasitic infection, correct nutrition, plant extracts with parasiticidal activity, biological control through nematophagous fungi and adequate management and hygiene of the pastures and stables, for example by regular removal of feces. Quarantine practices, chain harrowing and mowing pastures, rotating pastures and mixing or alternating grazing host species are other examples of good management practices effective in reducing parasite transmission (Lyons et al., 1999; Nielsen & Reinemeyer, 2018a).

3.3 STRONGYLID IDENTIFICATION METHODS

The horse health is globally affected by a variety of parasitic helminths. Strongylids are considered the most important parasitic nematodes due to the impact of co-infections and development of anthelmintic resistance. This requires improved diagnostic tools to identify and differentiate the spectrum of parasite species present, to identify resistant species or attribute pathological effects to individual species or species combinations. With a few exceptions, strongylids are difficult to distinguish at their larval stages and still require an expert to discern species in the adult stage. To complicate the situation, the validity of some cyathostomins is questionable and others might represent cryptic species complexes. Since the co-infection with cyathostomins is rather a rule than an exception, several methods have been developed to overcome these limitations (Bredtmann et al., 2017).

3.3.1 MORPHOLOGICAL IDENTIFICATION

Historically, cyathostomins have been difficult to identify to species level and were morphologically described using 93 different names (Lichtenfels, Kharchenko, Krecek, & Gibbons, 1998). Since some were considered synonyms, there are currently 50 cyathostomin species recognized as valid (Lichtenfels et al., 2008). Identification keys were published summing up the descriptions (Tolliver, 2000; Lichtenfels et al., 2008). Strongylid eggs, L1 and L2 cannot be differentiated. Using L3, it is possible to identify some genera such as *Strongylus*, *Triodontophorus*, *Gyalocephalus* and *Poteriostomum* (Bevilaqua, Rodrigues, & Concordet, 1993), and considering the number, shape and arrangement of the intestinal cells of cyathostomin L3s, they can be divided into 8 types from A to H (Madeira de Carvalho et al., 2008; Kornaś et al., 2009; Santos, Madeira de Carvalho, & Molento, 2018). The classification is based on morphologic features of adults. It requires careful examination of characters at the anterior and posterior ends, such as size and shape of the buccal capsules, mouth collar, cephalic papillae, internal and external leaf-crown and their extra-chitinous supports, size and shape of the bursa, genital cone, gubernaculum and spicules, tail and different parts of the reproductive system (Lichtenfels, 1975; Lichtenfels et al., 2008). Since some characters are

very faint, it requires years of intensive training to properly identify strongylids morphologically, which is exclusive to a few experts worldwide (Lichtenfels et al., 2008). Furthermore, there have been controversies over the taxonomic status and the phylogenetic relationships of strongylids based on morphological characters (Gasser, Hung, Chilton, & Beveridge, 2004; Gao, Zhang, et al., 2017;).

3.3.2 MOLECULAR METHODS

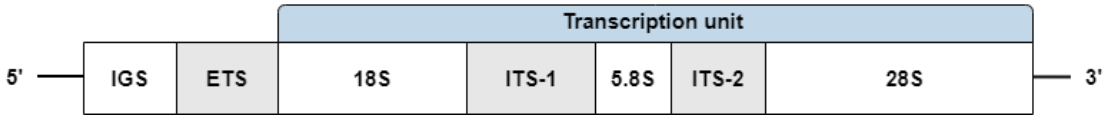
To overcome the limitations of morphological identification and allow improved insights into nematodes biology, epidemiology and pathogenicity, research has been focused on molecular identification. These methods target selected regions of the parasite genome which will allow identification of individual species within mixed infections, independently of the nematode life-stage. They also allow the genetic characterization of strongylid species for systematic studies (Bredtmann et al., 2017; Gao, Zhang, et al., 2017). Genomic DNA is stable, can be easily isolated, extracted and amplified by polymerase chain reaction (PCR) from frozen or alcohol fixed nematodes (Gasser, Chilton, Hoste, & Beveridge, 1993). Through PCR, minute quantities of DNA can be amplified from individual eggs, larvae and adult nematodes, providing opportunity to genetically compare all life cycle stages of different nematode species (Gasser et al., 1993; Chilton, 2004). Nevertheless, the ability to amplify DNA from small volumes increases the risk of microbial DNA contamination, which can be reduced by choosing more specific primers for PCR (Chilton, 2004).

Diverse molecular techniques have been employed to define genetic markers for the identification of strongylids, population genetics or evolutionary studies and diagnosis of infections (Nadler, 1990; Gasser et al., 1993; McManus & Bowles, 1996; Gasser et al., 1997; Chilton, 2004). A key element to the design of a sensitive and specific molecular tool is the selection of an appropriate target region of the parasite genome. Both nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) have been the target of these techniques. For the target regions being valid, DNA is extracted from morphologically distinct adult parasites, followed by PCR amplification and sequencing of the target regions. The DNA sequence generated for each species was compared to identify those which allow to detect individuals of closely related species without cross-reactivity with other equine parasitic nematodes (Hodgkinson, 2006).

The nuclear rDNA genes have a repetitive structure, i.e. they are organized in clusters of sometimes several hundred repeats localized in specific chromosomes. Each rDNA gene consists of the intergenic non-transcriber spacer (IGS), the external transcriber spacer (ETS) and the transcription unit comprising the genes coding for the 18S, 5.8S and 28S rRNA, which are separated by the first and second internal transcribed spacers (ITS-1 and ITS-2) (Chilton, 2004; Hodgkinson, 2006) (Figure 5). Within the rDNA gene, the sequences of both internal

transcribed spacers (ITS) and IGS have been used during the last decades as genetic markers for species identification and phylogenetic studies of strongylids (Hung, Gasser, Beveridge, & Chilton, 1999; Gasser & Newton, 2000; Chilton, 2004; Cwiklinski, Kooyman, Van Doorn, Matthews, & Hodgkinson, 2012). A characteristic of the ITS of bursate nematodes is that there is no evidence of sex-specific or life cycle stage-specific sequences (Chilton, 2004). Each species possesses comparatively unique ITS-1 and ITS-2 sequences. Occasionally, it is possible to find sequence polymorphism among rDNA gene paralogues of a single individual nematode, and low-level sequence polymorphism was detected within individuals of a species, indicating the presence of variable copies within the rDNA gene array (Elder & Turner, 1995; Chilton, 2004). This finding was in accordance with the theory of 'concerted evolution' (Elder & Turner, 1995), where repetitive rDNA gene sequences usually have a strong tendency to maintain homogeneity within a species. Due its lower level of intraspecific polymorphism, ITS sequences of nuclear rDNA genes remain an excellent tool for DNA diagnostics by quickly distinguishing between known species (Blouin, 2002). Additionally, nuclear genes are likely to be more useful for phylogenetic analysis than mitochondrial loci because they are not subject to lineage sorting.

Figure 5 – Example of an rDNA cluster (original). Gene scaling is only approximate.



Regarding mtDNA, the complete circular mitochondrial genome of strongylids contains 12 protein-coding genes (cytochrome c oxidase subunit 1-3 (COI-III); NADH dehydrogenase subunits 1-6 and 4L (NAD1-6 and NAD4L); cytochrome *b* (cytb); and Fo ATPase complex subunit 6 (atp6)), two rRNA genes and 22 transfer RNA (Xu et al., 2015; Gao, Liu, et al., 2017; Y. Gao, Qiu, et al., 2017; Gao, Zhang, et al., 2017; Qiu et al., 2018). The mtDNA evolves independently of the nuclear genome and it's considered to be inherited maternally, but not exclusively (Chinnery & Hudson, 2013). In nematodes, mtDNA evolves very quickly and the substitution rates varies among mitochondrial loci (Blouin, Yowell, Courtney, & Dame, 1998; Denver, Morris, Lynch, Vassilieva, & Thomas, 2000). The mitochondrial protein-coding gene group NADH dehydrogenase is considered to show relatively low conservation, while mitochondrial cytochromes show high conservation (Blouin et al., 1998). Blouin et al. (1998) recommend the use of more conserved mtDNA coding genes, such as COI, to study the phylogeny of closely related species. However, NAD4 is preferred for population genetic studies and identifying cryptic species in cases where other genetic markers show insufficient variation (Blouin et al., 1998).

The fact that mitochondrial genes evolve more quickly than ITS of the rDNA genes, by having higher substitutions rates and a high frequency of intraspecific polymorphism, makes them a less suitable target for quick diagnostic tests. Nonetheless, given samples of just a few individuals, mtDNA sequences are more useful for distinguishing among closely related species and detect cryptic species (Morgan & Blair, 1998; Blouin, 2002).

The COI gene is one of the most popular genetic markers since the development of universal primers from a research led by Dr. Folmer, that enabled amplification of a 710 bp COI region from a wide range of metazoan invertebrates (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994). This called “Folmer” partition of the COI gene is commonly used as barcode region across the animal kingdom (Hebert, Ratnasingham, & de Waard, 2003). Regarding nematodes, however, these universal Folmer primers generally give very poor amplification results, in particular for marine and parasitic species (De Ley et al., 2005; Bhadury et al., 2006; Derycke, Vanaverbeke, Rigaux, Backeljau, & Moens, 2010). New primers have been developed and tested to overcome this problem (Derycke et al., 2010; Prosser, Velarde-Aguilar, León-Règagnon, & Hebert, 2013; Duscher, Harl, & Fuehrer, 2015; Malysheva, Efeykin, & Teterina, 2017).

In addition, ITS-2 regions revealed a lack of phylum-wide primers, since the obtained sequences are usually very variable in size, making it difficult to align them. This fact, precluded the use of the ITS-2 as a universal nematode identification marker capable of analysis on high-throughput platforms (Floyd, Abebe, Papert, & Blaxter, 2002; De Ley et al., 2005).

Several molecular techniques have been used to identify and distinguish cyathostomins from nuclear rDNA or mtDNA markers, such as PCR-linked restriction fragment length polymorphism (PCR-RFLP) (Campbell, Gasser, & Chilton, 1995), PCR-linked single strand-conformation-polymorphism (SSCP-PCR) (Gasser & Monti, 1997), PCR-enzyme linked immunosorbent assay (PCR-ELISA) (Hodgkinson et al., 2003, 2005) and Reverse-Line-Blot-Assay (RLB) (Traversa et al., 2007; Ionita et al., 2010; Kooyman, van Doorn, Geurden, & Wagenaar, 2016).

3.3.3 SEROLOGICAL METHODS

The coproscopic diagnosis of larval cyathostominosis is difficult due the absence of adults expelling eggs (Murphy & Love, 1997). This has led to the study of alternative approaches such as serology to detected prepatent strongylid infections based on antigen-specific immunoglobulins (Ig). Equine IgG possesses four well-defined sub-isotypes designated IgGa, IgGb, IgGc and IgG(T). Two antigen complexes, only displayed to the immune system by larvae, have been identified and they showed potential as markers for estimating burdens of encysted cyathostomin larvae (Dowdall et al., 2002; Dowdall, Proudman, Love, Klei, & Matthews, 2003; Dowdall, Proudman, Klei, Mair, & Matthews, 2004). The anti-larval IgG(T)

serum responses for both antigen complexes, appeared to have good immunodiagnostic potential for prepatent cyathostomin infections (Dowdall et al., 2002). After purification of the antigen complexes, significant increases in serum IgG(T) levels to the complexes were observed in experimentally infected ponies in comparison to helminth-naïve ponies, reducing the likelihood of creating false positive results (Dowdall et al., 2003). Furthermore, when serum IgG(T) levels were analyzed in horses naturally and experimentally infected, there were significant correlations between the serum IgG(T) level and the burden of encysted cyathostomins. Additionally, in the serum from horses with suspicion of larval cyathostominosis, a significant increase in antigen-specific IgG(T) levels was detected (Dowdall et al., 2004). A protein component of one of these antigen complexes, designated cyathostomin gut-associated larval antigen-1 (Cy-GALA-1), was identified, isolated and the resultant recombinant protein (rCy-GALA-1) was shown to be a target of serum IgG(T) responses in naturally and experimentally infected horses (McWilliam, Nisbet, Dowdall, Hodgkinson, & Matthews, 2010). Segments of the Cy-GALA gene were isolated from four common cyathostomin species and the associated proteins expressed in recombinant form. An ELISA was developed based on the recombinant Cy-GALA proteins, allowing the detection of the immune response to cyathostomin larvae. No cross-reactivity to other parasites such as *Parascaris equorum*, *S. edentatus*, *S. vulgaris* and *Strongyloides westeri* was observed (Mitchell et al., 2016). This ability to detect prepatent cyathostomin infections could be available in the future as a routine diagnostic test to exclude or confirm larval cyathostominosis in horses with unspecific symptoms of colic. However, it does not provide information on the role of individual cyathostomin species in this syndrome, due to the absence of these serological methods to discriminate between different cyathostomin species.

3.3.4 PROTEOMICS METHODS

Proteomics is defined as the large-scale characterization of proteins. These proteins can be derived from a cell line, tissue, or organism and the goal of proteomics is to obtain a more global and integrated view of biology by studying all the proteins of a cell rather than each one individually (Graves & Haystead, 2002). Mass spectrometry (MS) has become an essential tool in many science fields (Awad, Khamis, & El-Aneed, 2015). Mass spectrometry methods measure the mass-to-charge ratio (m/z) values of the charged molecules and tandem mass spectrometric (MS/MS) fragments. The ability of MS analyzing proteins and other biological extracts is due to the development of soft ionization techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) that can transform biomolecules into ions. MALDI has the advantage of producing singly charged ions of peptides and proteins, minimizing spectral complexity. Regardless of the ionization method, the sensitivity of a mass spectrometer is related to the mass analyzer where ion separation occurs. Both quadrupole and time of flight (ToF) mass analyzers are commonly used and they can be configured

together as QToF tandem mass spectrometric instruments. Joining a quadrupole and time of flight analyzer resulted in the production of high-resolution mass spectrometers (i.e., Q-ToF). Tandem mass spectrometry (MS/MS), as the name indicates, is the result of performing two or more sequential separations of ions usually coupling two or more mass analyzers. This method is important to identify unknown proteins (Awad et al., 2015; El-Aneed, Cohen, & Banoub, 2009).

MALDI-TOF MS is widespread due to its cost-effectiveness, rapidness, accuracy and reliability. The technique involves generation of MALDI MS spectra for a given species and then matching it with the spectra database containing data for many well-defined species, deducing the species information by simple pattern matching algorithms (Murugaiyan & Roesler, 2017). It is a well-established technique for microbial species identification and has been successfully applied to a variety of parasites and their vectors. It was used in the past two decades to differentiate species of protozoan parasites (e.g. *Leishmania*, *Giardia*, *Cryptosporidium*), arthropods (e.g. *Culicoides*, *Glossina*, ticks), plant parasitic nematodes and more recently, animal parasitic nematodes, such as *Trichinella* (Perera, Vanstone, & Jones, 2005; Perera, Jones, Taylor, & Vanstone, 2009; Ahmad, Gopal, & Wu, 2012; Hoppenheit et al., 2013; Mayer-Scholl et al., 2016; Singhal, Kumar, & Virdi, 2016; Murugaiyan & Roesler, 2017). This method could also be applied to cyathostomin species identification, and a preliminary study using MALDI-TOF MS revealed distinct patterns for adult individuals of different species of cyathostomins (Bredtmann et al., 2017; Bredtmann et al., 2019). A database needs to be generated from morphologically and molecularly identified specimens. However, it requires further research to overcome possible limitations, such as different spectra elicited by different development stages.

CHAPTER 4: MATERIALS AND METHODS

4.1 RESEARCH AIMS AND OBJECTIVES

The present study addresses some of the gaps in our knowledge on the subfamily Cyathotominae. The primary aims of this study were to verify the utility of two different genes as barcoding regions on six different species of cyathostomins (*Cylicocyclus nassatus*, *Coronocyclus labiatus*, *Coronocyclus coronatus*, *Cylicostephanus longibursatus*, *Cylicostephanus minutus* and *Cylicostephanus calicatus*) collected from different equid host species; and to evaluate the phylogenetic relationships between these cyathostomin species and genera.

For this purpose, the goals of this work were:

- to obtain, through molecular methods, two different genomic sequences (rDNA ITS-2 and mtDNA COI) from 6 morphologically different cyathostomin species, collected from different equid host species;
- to determine and evaluate a maximum likelihood phylogenetic tree for each gene and for a combined data set;
- to compare the genetic variation for both genomic sequences within each cyathostomin specie, between species and other observed groups.

4.2 SAMPLES

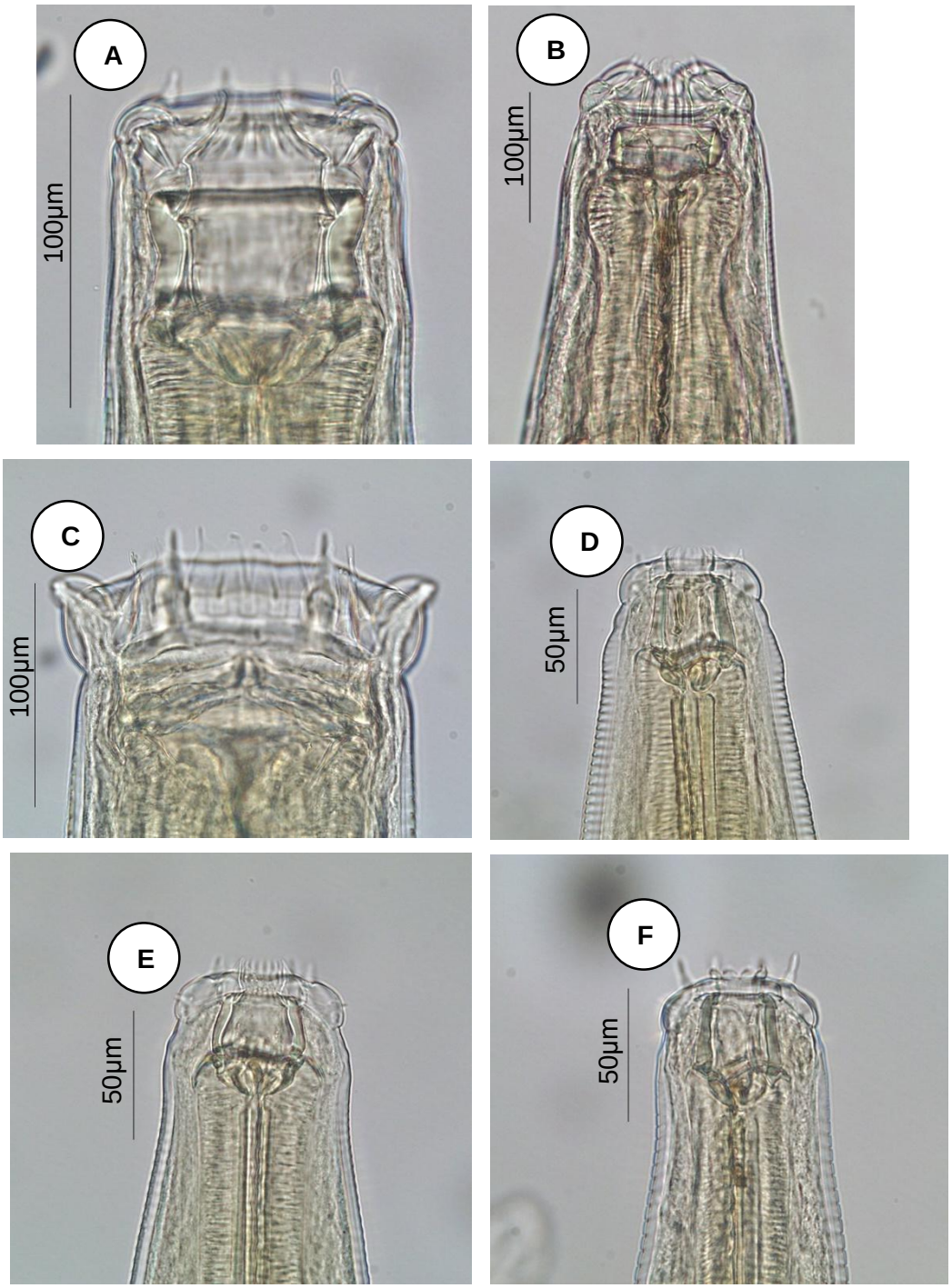
Adult worms were collected from eight (without defined breed) German horses (*Equus ferus caballus*) during necropsy and after deworming with 0.2% aversectin C (“Univerm”, PharmBioMed, Moscow, Russia) from fecal samples of five species of equines kept at the Askana Nova Biosphere reserve, Ukraine, i.e. a domestic horse (*Equus ferus caballus*), a wild Przewalski's horse (*Equus ferus przewalskii*), a donkey (*Equus asinus*), a Turkmenian kulan (*Equus hemionus kulan*) and a plain zebra (*Equus burchelli*) (Figure 6). The worms were washed in distilled water and fixed in 70% ethanol. Individual worms were identified to species based on their morphological characteristics, according to Lichtenfels et al. (2008). Specimens identified as *Cylicocyclus (Cyc.) nassatus*, *Coronocyclus (Cor.) labiatus*, *Coronocyclus (Cor.) coronatus*, *Cylicostephanus (Cys.) longibursatus, Cylicostephanus (Cys.) minutus* and *Cylicostephanus (Cys.) calicatus* were included in this study (Figure 7). For each equid host, up to ten adult individuals (usually five males and five females) of each these cyathostomin species were selected.

Figure 6 - Host species' examples (original; pictures from Germany – Berlin Zoo (A) and Berlin Tierpark (B and C) - and Portugal - Guarda (D and E)).

4.3 DNA EXTRACTION

Genomic DNA was isolated from the debris of individual worms that remained after protein extraction, using the NucleoSpin® Tissue XS kit protocol (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations. The protocol has recently been published by (Bredtmann et al., 2019). DNA samples were stored at -20ºC for further experiments.

Figure 7 - Cyathostomins included in the study. A - *Coronocyclus coronatus*, B- *Coronocyclus labiatus*, C - *Cylicocyclus nassatus*, D - *Cylicostephanus calicatus*, E - *Cylicostephanus longibursatus* and F - *Cylicostephanus minutus* (source: adapted from Madeira de Carvalho, 2002).



4.4 POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION

PCRs targeting the internal transcribed spacer 2 (ITS-2) and a partial cytochrome oxidase I (COI) fragment were conducted using a high-fidelity DNA polymerase (Phusion II, Thermofisher Scientific). The rDNA ITS-2 region was amplified with NC1 and NC2 primers. The mtDNA COI region was amplified using the primers COI_Nema_Fw and COI_Nema_Rv. The primers used can be found in Table 3.

Table 3 - Primers used on the rDNA and mtDNA amplification

Region	Primer name	Sequence	Approximate fragment size	Reference
rDNA ITS-2	NC1 (forward)	5'-ACGTCTGGTTCAGGGTTGTT-3'	400bp	Gasser, Chilton, Hoste, & Beveridge, 1993
	NC2 (reverse)	5'-TTAGTTTCTTTTCCTCCGCT-3'		
mtDNA COI	COI_Nema_Fw (forward)	5'-GAAAGTTCTAATCATAARGATATTGG-3'	650bp	Duscher, Harl, & Fuehrer, 2015
	COI_Nema_Rv (reverse)	5'-ACCTCAGGATGACCAAAAAAYCAA-3'		

Amplification reactions using both pairs of primers were carried out in a final volume of 20 µL, including 12.4 µL of nuclease free water (DEPC treated water), 4.0 µL of HF Buffer (Thermofisher Scientific), 0.4 µL of deoxyribonucleotide triphosphates (dNTP) mix (10 µM) (Thermofisher Scientific), 0.2 µL (0.4U) of Phusion Hot Start II High-fidelity DNA polymerase (Phusion II, Thermofisher Scientific), 0.5 µL of each primer (10 µM) and 2 µL of genomic DNA. During every PCR run, negative and positive control samples were included: negative controls did not include DNA, while positive controls contained plasmid DNA from cyathostomins (COI) or from *Trichostrongylus colubriformis* (ITS-2). Thermal cycling had an initial denaturation at 98 ºC for 30 s, followed by 40 cycles of denaturation at 98 ºC for 10 s, annealing at 55ºC for 30 s and extension at 72ºC for 30 s. Finally, an extension at 72 ºC for 5 min.

A 12 µL mixture containing 8 µL of sterile water, 2 µL of PCR product and 2 µL of 6× DNA loading dye (Thermofisher Scientific) was loaded into the wells of an 1.5% agarose gel in TRIS-Acetate-EDTA (TAE) buffer and stained with GR Green (Labgene, Châtel-Saint-Denis, Switzerland). A 100 bp ladder was used as DNA size marker in each gel for estimating the size of the amplicons. The DNA fragments resulting from the PCR were then separated according to their size using agarose gel electrophoresis. The gels were photographed under UV illumination. When presenting a single band, the PCR products were purified with DNA Clean & Concentrator kit (Zymo Research, Freiburg, Germany). When double bands were present, Gel DNA Recovery kit (Zymo Research, Freiburg, Germany) was used to recover both bands. For this purpose, a second agarose gel electrophoresis, as described above, was used to cut-out bands under blue-light illumination followed by a third electrophoresis after purification to confirm the success of the PCR purification procedure and estimate the DNA concentration. The purified products were stored at -20ºC until further use.

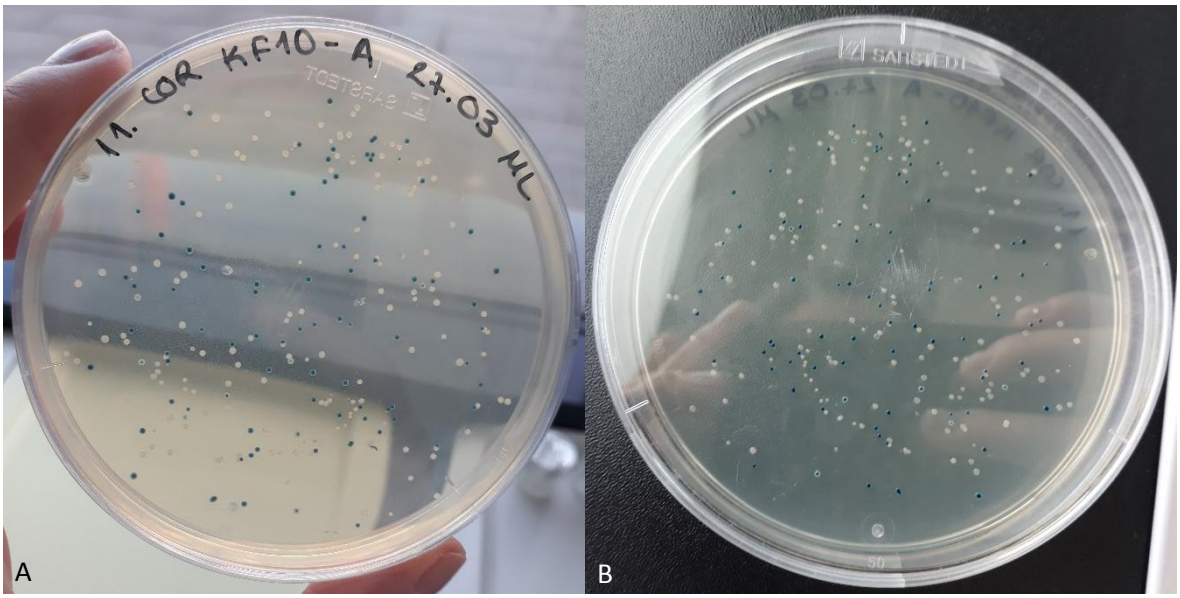
4.5 MOLECULAR CLONING

The sequences were determined after cloning of the fragments into pSC-B-amp/kan vector using the StrataClone Blunt PCR Cloning Kit (Agilent Technologies, Waldbronn, Germany), according to the following protocol. The first step consisted of the ligation of the purified DNA fragment (0.5 µL) into the vector (mix of 0.75 µL of Cloning Buffer with 0.25 µL of Vector Mix). The mixture was left incubating for 30 min at room temperature. The following steps resulted in transformation of plasmid vectors as episomes into the bacterial cells. For this, 0.5 µL of the previous ligation mixture were added to 12.5 µL of competent *Escherichia coli* (*E. coli*) cells from the StrataClone SoloPack Competent Cells (Agilent Technologies, Waldbronn, Germany), kept on ice for 30 min, then heat-shocked at 42 ºC for 45 s, and again placed on ice for 2 min. The next step was to add 65 µL of pre-warmed (42ºC) Lysogeny Broth (LB) medium to the cells and incubate for 1 hour at 37 ºC and shaking at 200 rpm.

Subsequently, the cells were plated on LB agar plates containing 100 µg/ml Carbenicillin and 20 µg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), and left incubating over night at 37 ºC. The vector used contains the β-lactamase (bla) gene, which confers resistance to ampicillin and carbenicillin (chosen for being more stable) (Sambrook & Russell, 2001), allowing the transformed cells to grow in this medium. Additionally, the vector encodes the LacZ α-peptide, which encodes a small part of the β-galactosidase enzyme. This α-peptide is able to combine with the much larger β-fragment of LacZ encoded in the bacterial genome to form a functional β-galactosidase. This enzyme is responsible for hydrolyzing the X-gal, resulting in a final blue product. Its expression occurs when the transformed cell did not incorporate the PCR product, resulting in the grow of blue colonies in the presence of X-gal (Sambrook & Russell, 2001). When a cell is correctly transformed with the recombinant plasmid, it splits the LacZ α-peptide and incorporates the PCR product in between, resulting in growth of white colonies (Figure 8).

The next step was the propagation of the *E. coli* cells, by picking a white colony from the agar plate, inoculate it in a test tube containing 5 mL of LB medium with 100 µg/ml Carbenicillin and incubate the culture overnight at 37 ºC and 200 rpm. Finally, the plasmid of the liquid culture was isolated using the GenUp™ Plasmid Kit (biotechrabbit™, Berlin, Germany) according to the manufacturer's protocol.

Figure 8 - LB agar plates with colonies containing either a recombinant plasmid (white) or a plasmid without insert (blue). A – bottom view; B – top view (original)



4.6 EcoRI DIGESTION

Since the vector also contains the specific recognition sequence for the EcoRI endonuclease flanking the sites for the insertion for the PCR product, the EcoRI restriction enzyme was used to verify the presence and the estimated size of the DNA fragment. The protocol used included a mix of 16.5 µL of double-distilled water, 2 µL of 10× Buffer EcoRI (Thermo Fisher Scientific), 0.5 µL of EcoRI (Thermo Fisher Scientific) and 1 µL of plasmid DNA. Reactions were incubated at 37ºC for 1 hour. Then, 4 µL of 6× DNA loading buffer (Thermo Fisher Scientific) were added to the digested product and then 12 µL of this mixture were subjected to agarose electrophoresis in 1.5% agarose gels as described above. A 100 bp and 1000 bp ladder were used as DNA size markers in each gel for estimating the size of the inserts.

4.7 SEQUENCING

The DNA concentration of the isolated plasmid was determined using the Epoch™ Microplate Spectrophotometer (BioTek Instruments) and, if need, diluted with double-distilled water (final concentration approx. 100ng/µL). The plasmid with the insert were Sanger-sequenced on both strands at LGC Genomics (Berlin, Germany), using the primer “M13-24 F blue” (5''-GTAAAACGACGGCCAGTGAGCGCG-3'') or “M13-24 R blue” (5''-AACAGCTATGACCATGATTACGCC-3'').

4.8 PHYLOGENETIC ANALYSES

The sequences obtained in the study were manually edited using MEGA7 software (Kumar, Stecher, & Tamura, 2016) by removing the vector and primers. All ITS-2 sequences were aligned using the MAFFT method on the MAFFT online server (Katoh, Rozewicki, & Yamada, 2017) with default parameters and -G-INS-i as iterative refinement method. COI sequences aligned unambiguously with no gaps and did not need alignment, but the amino acid sequences were verified by translating the nucleotide sequences into protein sequences using the invertebrate mitochondrial genetic code on MEGA7.

Phylogenetic analysis were conducted using Maximum Likelihood algorithm on the IQ-TREE web server (Trifinopoulos, Nguyen, von Haeseler, & Minh, 2016). On this platform, the substitution models were determined with ModelFinder, which automatically uses Bayesian Information Criterion (BIC) to choose the best-fit model for each analysis (Kalyaanamoorthy, Minh, Wong, Von Haeseler, & Jermiin, 2017), and the FreeRate models with rate heterogeneity across sites model were included in the model consideration. Separate analyses were performed on the ITS-2 and COI genes, followed by a combined analysis on both genes. Optimal trees were obtained by partitioned analyses of both nuclear and mitochondrial markers. For the COI analysis, DAMBE and FASconCAt-G software was also used (Kück & Longo, 2014; Xia, 2018) to split COI sequences into different partitions for codon position 1 and 2 vs. codon position 3, which allowed fitting of different models for each partition. In the combined analysis, ITS-2 and two COI partitions were concatenated, and different models were chosen for each partition. Branch support analysis on the most likely tree were conducted on all consensus trees by ultrafast bootstrap analysis (Hoang, Chernomor, von Haeseler, Minh, & Le, 2007), with 1000 bootstrap replicates and Shimodaira-Hasegawa (SH) approximate likelihood ratio test. The bootstrap and SH values are quoted on the branches. The trees were visualized and edited using MEGA X and FigTree software (Rambaut, 2012; Kumar, Stecher, Li, Knyaz, & Tamura, 2018). According to Hung et al., (1999) cladograms, *Cys. minutus* was chosen to root the trees.

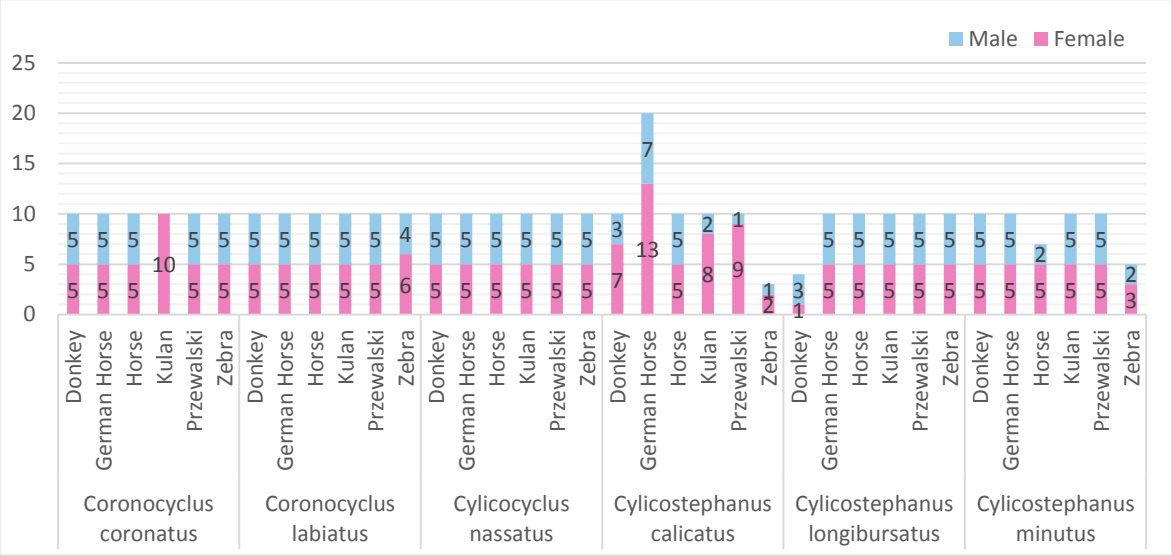
Identities between sequences were calculated for ITS-2 and COI genes using the R package ape (Analyses of Phylogenetics and Evolution) version 5.2 in R software version 3.5.0 (Paradis, Claude, & Strimmer, 2004; R Core Team, 2018). R software was accessed via R Studio version 1.1.463. The resulting matrix was manually converted to columns of identity between groups of sequences, according to the clusters observed in the trees. Box plots comparing the identity between groups were created with GraphPad Prism 8 software. Kruskal-Wallis test followed by a Dunn's multiple comparison test were used to identify significant differences in identity between different groups (GraphPadSoftware, 2018).

CHAPTER 5: RESULTS

5.1 SAMPLES

A total of 349 cyathostomin samples (279 from Ukraine and 70 from Germany) were collected. Regarding the hosts, 70 from German horse, while 54 worms were collected from a donkey, 57 from Ukrainian a horse, 60 from a Kulan, 60 from a Przewalski horse and 48 from a zebra at Askana Nova (Ukraine). Concerning the cyathostomin species, 60 adult worms of each *Coronocyclus coronatus*, *Coronocyclus labiatus* and *Cylicocyclus nassatus*, 63 of *Cylicostephanus calicatus*, 54 of *Cylicostephanus longibursatus,* and 52 of *Cylicostephanus minutus* were collected. The distribution of samples among species, hosts and sex as is shown in the Figure 9.

Figure 9 - Distribution of the cyathostomin samples.



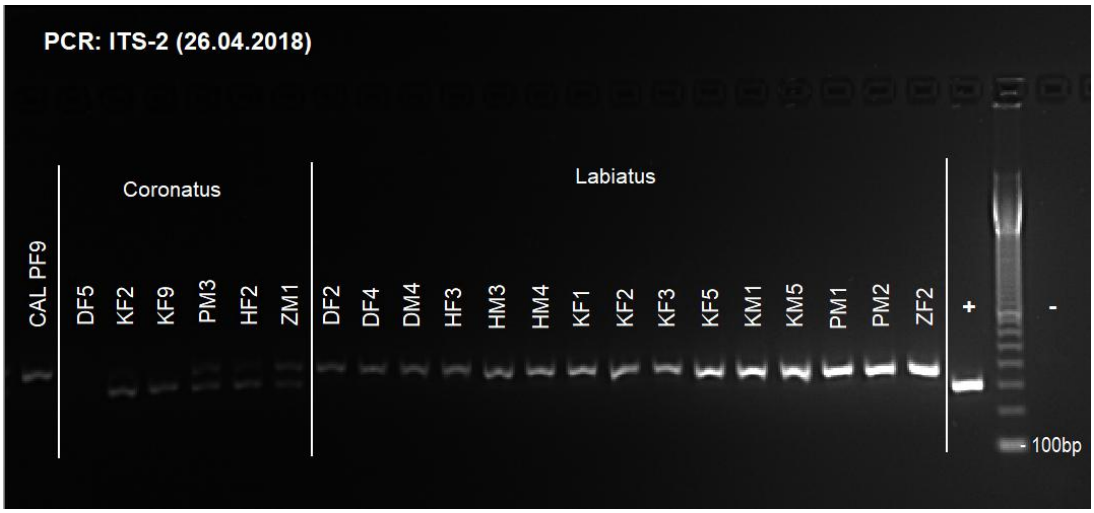
5.2 PCR AMPLIFICATION AND MOLECULAR CLONING

The DNA was successfully extracted from all worms. The PCR targeting the COI fragment was unsuccessful for 12 samples and, therefore, these samples were excluded from some of the analysis, including in particular the combined ITS-2 and COI tree. With this exception, the COI PCR was successful for all the remaining 337 samples. The resulting COI sequences after molecular cloning all consisted of 653 nucleotides, corresponding to 217 amino acids in the translated protein sequence, without the presence of stop codons, representing uninterrupted open reading frames, suggesting that they were all functional.

The PCR targeting the ITS-2 fragment was successful for all samples. During the documentation of the gels resulting from gel electrophoresis after PCR, the presence of two differently sized fragments (“double bands”) on some *Cor. coronatus* samples (Figure 10 and 11) was observed. Each of these fragments were successfully extracted, purified and cloned.

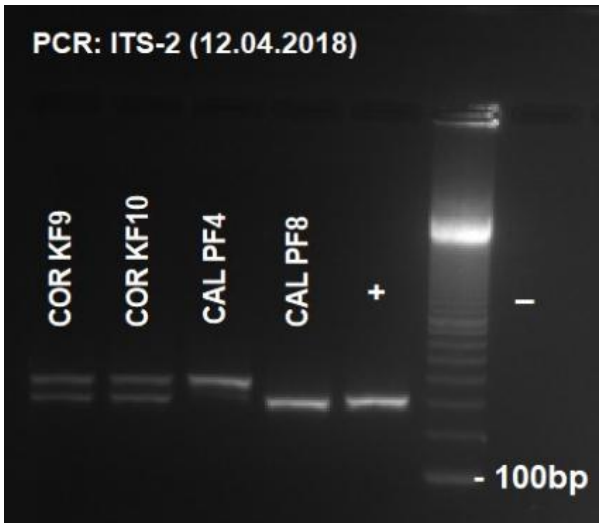
Furthermore, two differently size fragments on *Cys. calicatus* samples were found. However, unlike *Cor. coronatus,* each sample only contained one of the fragments (Figure 11). The deletion between the larger and smaller sequences encompassed the positions 108-196 (89bp). The size and number of the ITS-2 sequences for each cyathostomin species determined by molecular cloning can be found in Table 4.

Figure 10 - Picture of a gel electrophoresis' results of the rDNA region ITS-2 revealing the presence of double band on some *Cor. coronatus* samples (original).



Legend – *Cys. calicatus* (CAL), *Cor. coronatus* (Coronatus) and *Cor. labiatus* (Labiatus) samples are separated by the white vertical lines. The samples are identified by the following code: D, K, P, H and Z for the hosts donkey, kulan, Przewalski horse, Ukrainian horse and zebra, respectively; F/M for female or male; and a number indicating the individual specimen. Positive (+) and negative (-) controls.

Figure 11 - Amplified picture of a gel electrophoresis' results of the rDNA region ITS-2 revealing the presence of double band on *Cor. coronatus* samples (COR) and different sized bands on *Cys. calicatus* (CAL) (original).



Legend – CAL and COR represent the specie *Cys. calicatus* and *Cor. coronatus*. The samples are identified by the following code: K and P for the hosts kulan and Przewalski horse; F/M for female or male; and a number indicating the individual specimen. Positive (+) and negative (-) controls.

Table 4 - ITS-2 sequences size and number of sequences for each cyathostomin species.

Specie	Sequence size	Number of sequences
Coronocyclus coronatus	370 bp	36
	369 bp	5
	278 bp	60
Cylicostephanus calicatus	370 bp	45
	369 bp	1
	281 bp	17
Coronocyclus labiatus	368 bp	60
Cylicocyclus nassatus	370 bp	59
	365 bp	1
Cylicostephanus longibursatus	370 bp	54
Cylicostephanus minutus	265 bp	51
	266 bp	1

During the analysis on the ITS-2 sequences, it was noted that the larger fragments of *Cor. coronatus* (370 bp and 369 bp) had the same size and were visually identical to the larger fragments of *Cys. calicatus* (370 bp and 369 bp). This similarity extended to the smaller fragments of these two species. Because of this, a separate analysis was conducted, including only these two species, and a manuscript written, submitted and published to *Infection Genetics and Evolution* with the title “Nuclear and mitochondrial marker sequences reveal close relationship between *Coronocyclus coronatus* and a potential *Cylicostephanus calicatus* cryptic species complex” and the complete article can be found on Annex 1.

To simplify the present analysis, the larger ITS-2 fragments of *Cor. coronatus* (370 bp and 369 bp) were excluded from this analysis. The remaining 60 smaller sequences from *Cor. coronatus*, together with the ones from the other species, resulted in a total of 349 sequences for the ITS-2 gene.

5.3 PHYLOGENETIC ANALYSIS

All phylogenetic trees were compressed to allow a better viewing. The compressed trees based on the ITS-2, COI and concatenated sequences can be seen on Figures 12, 13 and 14, respectively. A guide to visualize the complete trees can be found on Annex 2.

Analyzing each tree, most sequences seemed to group by species. Within *Cys. minutus,* three clusters of ITS-2 sequences were obtained, the first consisted of only one sequence (MIN G F02), the second contained 13 sequences and the third was composed of 38 sequences which were considered as operational taxonomical units (OTU) I, II and III, respectively. Similarly, the *Cys. minutus* COI sequences also grouped into 3 clusters, each presenting the same samples and named as above. One sample (MIN Z F03), however, present in the OTU II of the ITS-2 tree, switched with another sample (MIN Z F02), being now present in the OTU III of the COI tree, and vice versa. Comparing both trees, the major difference is in the *Cys. minutus* OTU I, which diverged closer to the *Cys. longibursatus* cluster on the COI tree. Also, on the tree from the concatenated sequences, *Cys. minutus* presented 3 clusters with the exact same sequences from the COI tree, but additionally, sequence MIN Z F03 appeared separated supported by a high bootstrap value. All trees present high bootstrap values for the three *Cys. minutus* clusters.

Concerning *Cyc. nassatus*, three clusters were obtained for each locus and the concatenated data set. The first to diverge on all trees is constituted by only one sample (NAS P F01), supported by a bootstrap value of 97% (later identified as *Cyc. ashworthi)*. The second cluster is also constituted by only one sample. However, in this case, the sample differs between trees, being NAS H M04 in the ITS-2 tree and NAS D F02 for the tree with COI and concatenated sequences. The last and biggest cluster is composed by the rest of the *Cyc. nassatus* sequences.

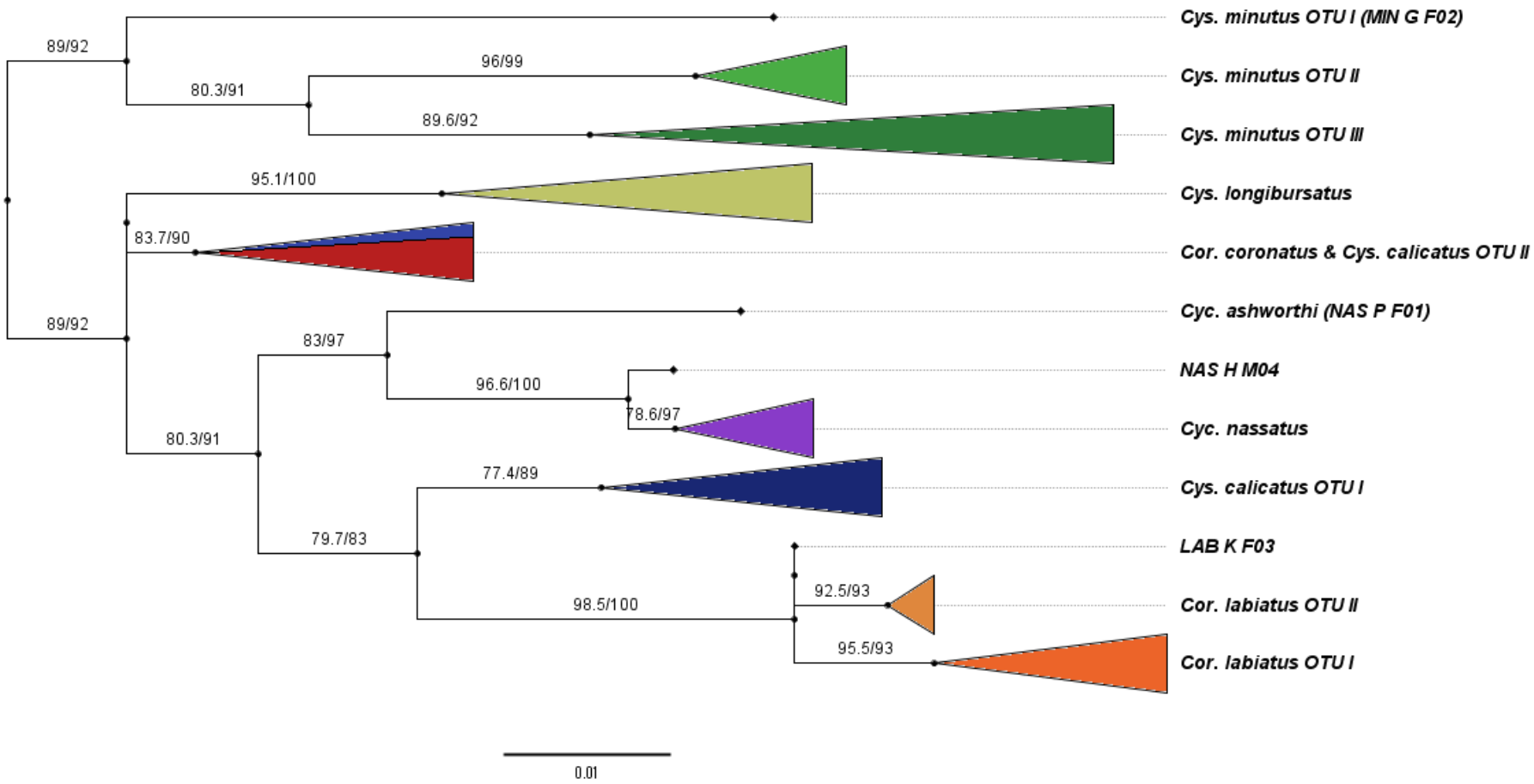
Regarding *Cor. labiatus,* three clusters were observed for the ITS-2 and COI sequences. On the ITS-2 tree, these branches appeared as a polytomy with a high bootstrap value. The smaller cluster is constituted by only one sample on both trees, but with a different sample. The other two main clusters observed are supported by high bootstrap values and were considered as different OTU's. On each locus tree, the biggest cluster was designated as OTU I, and the smaller, with 14 ITS-2 and 17 COI sequences, as OTU II. It was observed that diverse mitochondrial and nuclear haplotypes were mixing with each other through the different clusters. The tree from the concatenated sequences showed a different topology, but it was possible to distinguish two major clusters, which were designated as above.

Comparatively, *Cys. longibursatus* samples were more homogenous and presented a single cluster on all trees. The internal branches showed low bootstrap values on the ITS-2 tree, but higher values for the other trees.

Cor. coronatus sequences gathered in a single cluster on all trees, but its position varied from each tree. The ITS-2 sequences presented themselves as a polytomy, with a smaller number of internal branches which included the smaller 17 *Cys. calicatus* ITS-2 sequences. In the trees based in the COI and concatenated sequences, *Cor. coronatus* samples appeared between distinct branches of *Cys. calicatus* samples and revealed more heterogeneity through the presence of different mitochondrial haplotypes.

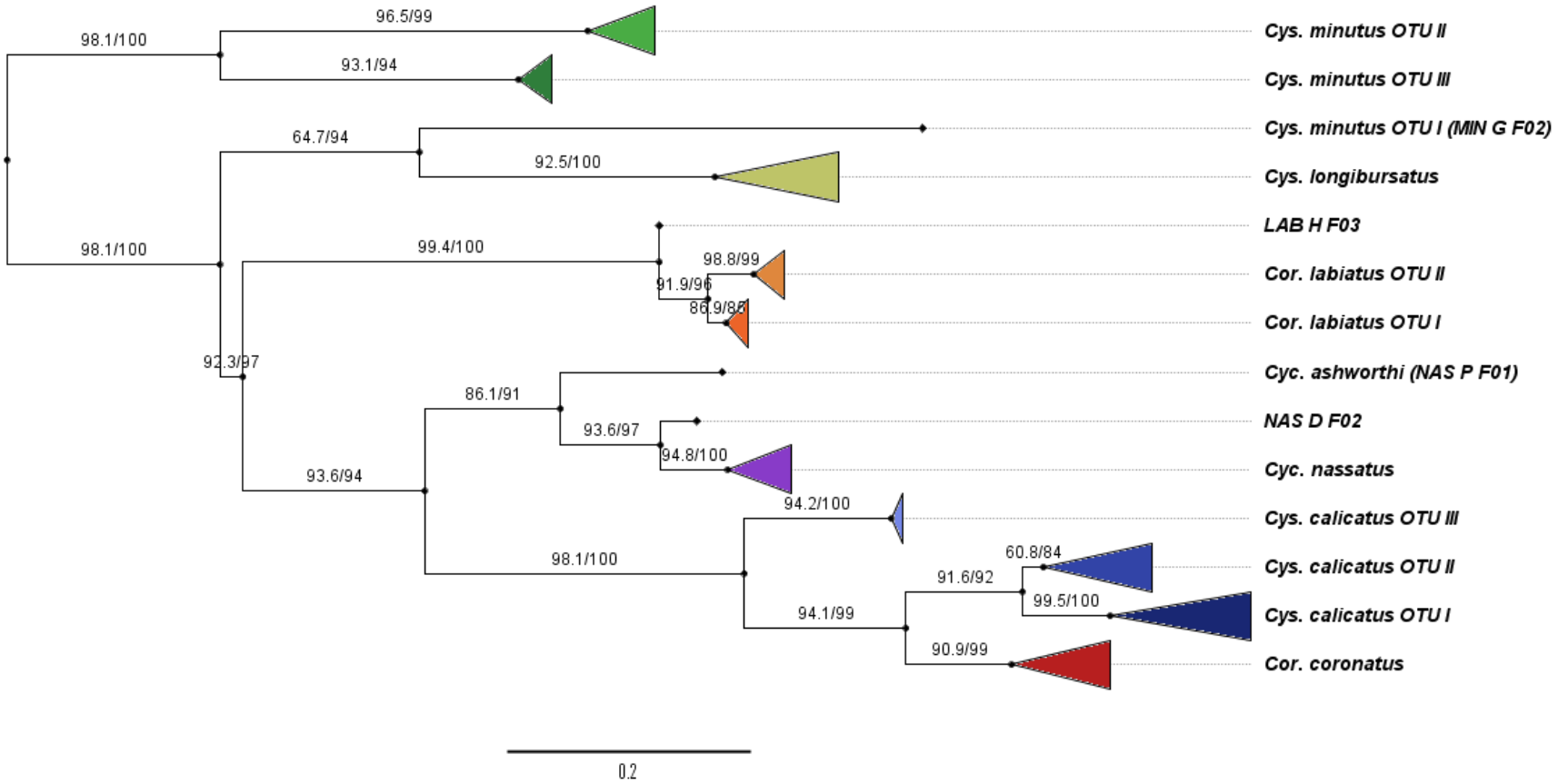
Within *Cys. calicatus*, two distinct clusters were obtained from ITS-2 sequences. The cluster composed by the larger sequences, was designated as OTU I, while the other, composed by the smaller ITS-2 sequences and which clustered together with the *Cor. coronatus*, was considered as OTU II. The internal branches of the first OTU presented evidence of different and distinct nuclear haplotypes. Regarding the COI sequences, three clusters were formed. The first diverging cluster is constituted only by two samples and designated as OTU III. This cluster is also present with the same samples on the tree from the concatenated sequences and was given the same designation. A second cluster is formed with the same samples found together with *Cor. coronatus* on the ITS-2 tree and was named accordingly (*Cys. calicatus* OTU II). These samples are also present together on the combined tree, forming a cluster from which the *Cor. coronatus* samples derivate, and was named accordingly as above. The remaining samples grouped together, forming a larger cluster designated as OTU I. An internal branch from this cluster, derives separately on both COI and combined tree, being composed of the same 23 samples. On the COI tree, various mitochondrial haplotypes are present.

Figure 12 - Maximum likelihood phylogenetic condensed tree calculated using aligned ITS-2 sequences.



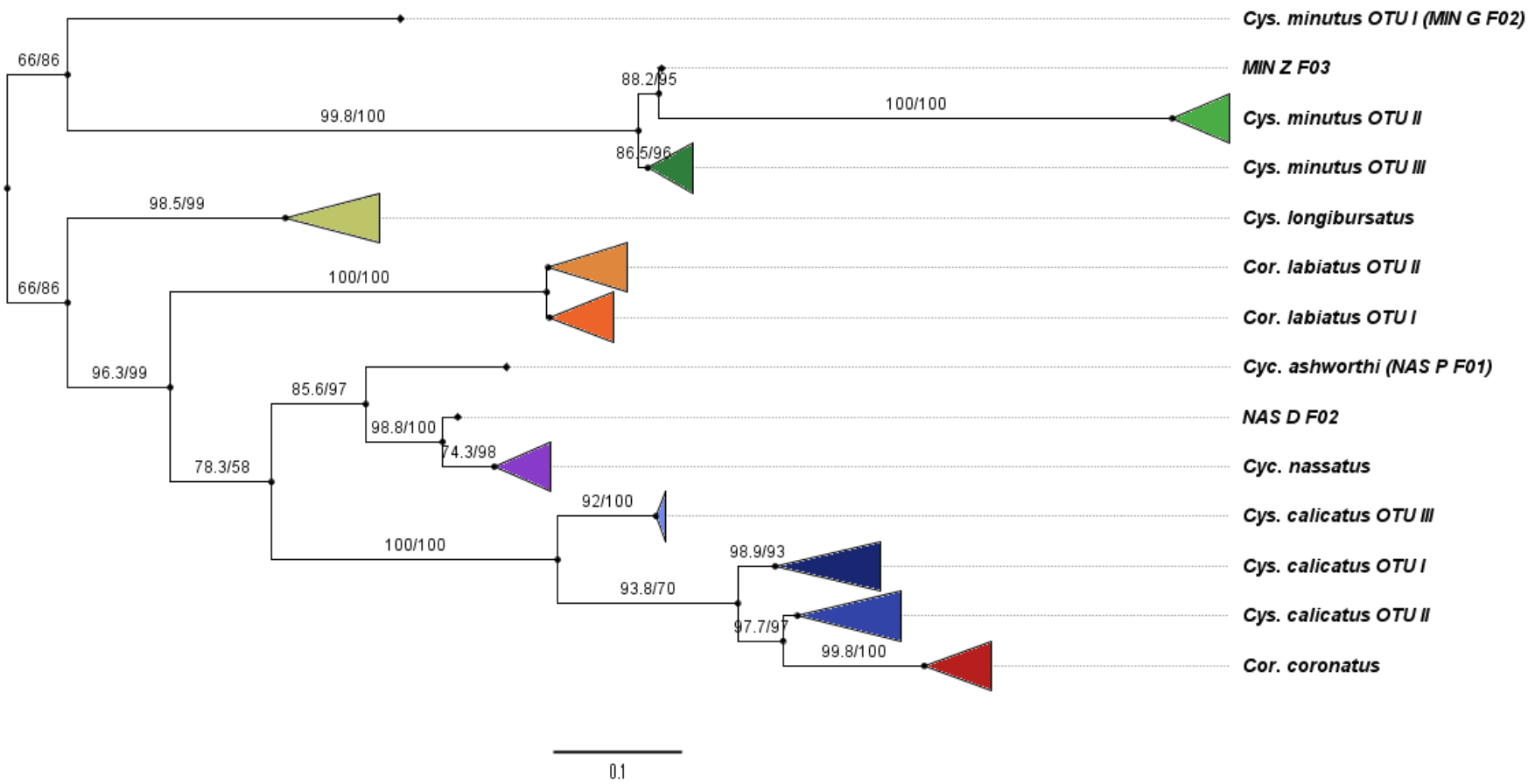
Legend – The scale bar represents 0.01 substitutions per site and node support was obtained by ultrafast bootstrapping (1000 replicates) after and the Shimodaira-Hasegawa likelihood ratio test before the slash. Individual specimen that were not assigned to one of the major clades are designated according to the following code: MIN/ NAS/ LAB/ LON/ COR/ CAL for the species *Cys. minutus*, *Cyc. nassatus*, *Cor. labiatus*, *Cys. longibursatus*, *Cor. coronatus* and *Cys. calicatus*, respectively; G, H, P, K, D, Z for the hosts German horse, Ukrainian horse, Przewalski's horse, kulan, donkey and zebra, respectively; F/M for female or male; a number indicating the individual specimen.

Figure 13 – Maximum likelihood phylogenetic condensed tree calculated using aligned COI sequences.



Legend – The scale bar represents 0.2 substitutions per site and node support was obtained by ultrafast bootstrapping (1000 replicates) after and the Shimodaira-Hasegawa likelihood ratio test before the slash. Individual specimen that were not assigned to one of the major clades are designated according to the following code: MIN/ NAS/ LAB/ LON/ COR/ CAL for the species *Cys. minutus*, *Cyc. nassatus*, *Cor. labiatus*, *Cys. longibursatus*, *Cor. coronatus* and *Cys. calicatus*, respectively; G, H, P, K, D, Z for the hosts German horse, Ukrainian horse, Przewalski's horse, kulan, donkey and zebra, respectively; F/M for female or male; a number indicating the individual specimen.

Figure 14 – Maximum likelihood phylogenetic condensed tree calculated using aligned concatenated ITS-2 and COI sequences.



Legend – The scale bar represents 0.1 substitutions per site and node support was obtained by ultrafast bootstrapping (1000 replicates) after and the Shimodaira-Hasegawa likelihood ratio test before the slash. Individual specimen that were not assigned to one of the major clades are designated according to the following code: MIN/ NAS/ LAB/ LON/ COR/ CAL for the species *Cys. minutus*, *Cyc. nassatus*, *Cor. labiatus*, *Cys. longibursatus*, *Cor. coronatus* and *Cys. calicatus*, respectively; G, H, P, K, D, Z for the hosts German horse, Ukrainian horse, Przewalski's horse, kulan, donkey and zebra, respectively; F/M for female or male; a number indicating the individual specimen.

5.4 SEQUENCE IDENTITY ANALYSIS

The comparison of sequence identities between different species and some OTUs can be seen in Figure 15 for the ITS-2 sequences and in Figure 16 for the COI sequences. For this analysis, a comparison within all sequences of each species was included.

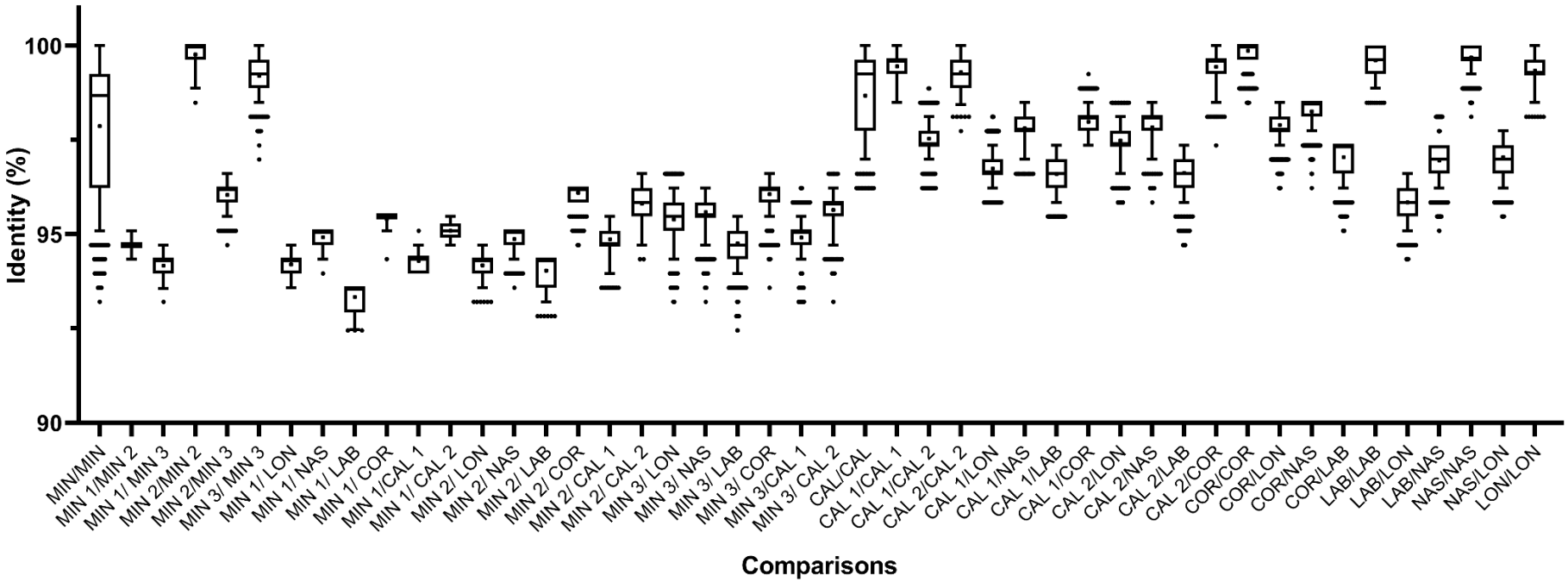
The operational taxonomical units from *Cys. calicatus* and *Cys. minutus* were considered distinct groups in each analysis and included in the comparison. The *Cyc. nassatus* sample NAS P F01 identified as *Cyc. ashworthi,* was excluded from this analysis.

Pairwise comparisons between all sequences belonging to *Cys. minutus,* revealed an intra-specific identity from 93.21 to 100% for the ITS-2 locus and from 86.87 to 100% for the COI locus, while within the three clusters it ranged from 96.98 to 100% and 94.18 to 100% for the ITS-2 and COI locus, respectively. In contrast, the identities between sequences of different *Cys. minutus* clusters ranged from 93.21 to 96.60% for the ITS-2 locus and from 86.37 to 89.74% for the COI locus.

Pairwise comparisons between all sequences belonging to *Cys. calicatus* revealed an intra-specific identity for the ITS-2 and COI locus between 96.23-100% and 86.68-100%, respectively, while within the clusters it ranged from 94.18 to 100% for the ITS-2 locus and from 91.12 to 100% from the COI locus. The identities between sequences from the different *Cys. calicatus* clusters ranged from 96.23 to 98.87% and 86.68 to 93.26% at the ITS-2 and COI locus, respectively. The identities between *Cor. coronatus* sequences and the *Cys. calicatus* OTU II cluster ranged, for the ITS-2 locus, from 97.36 to 100% and, for the COI locus, from 87.75 to 92.65%.

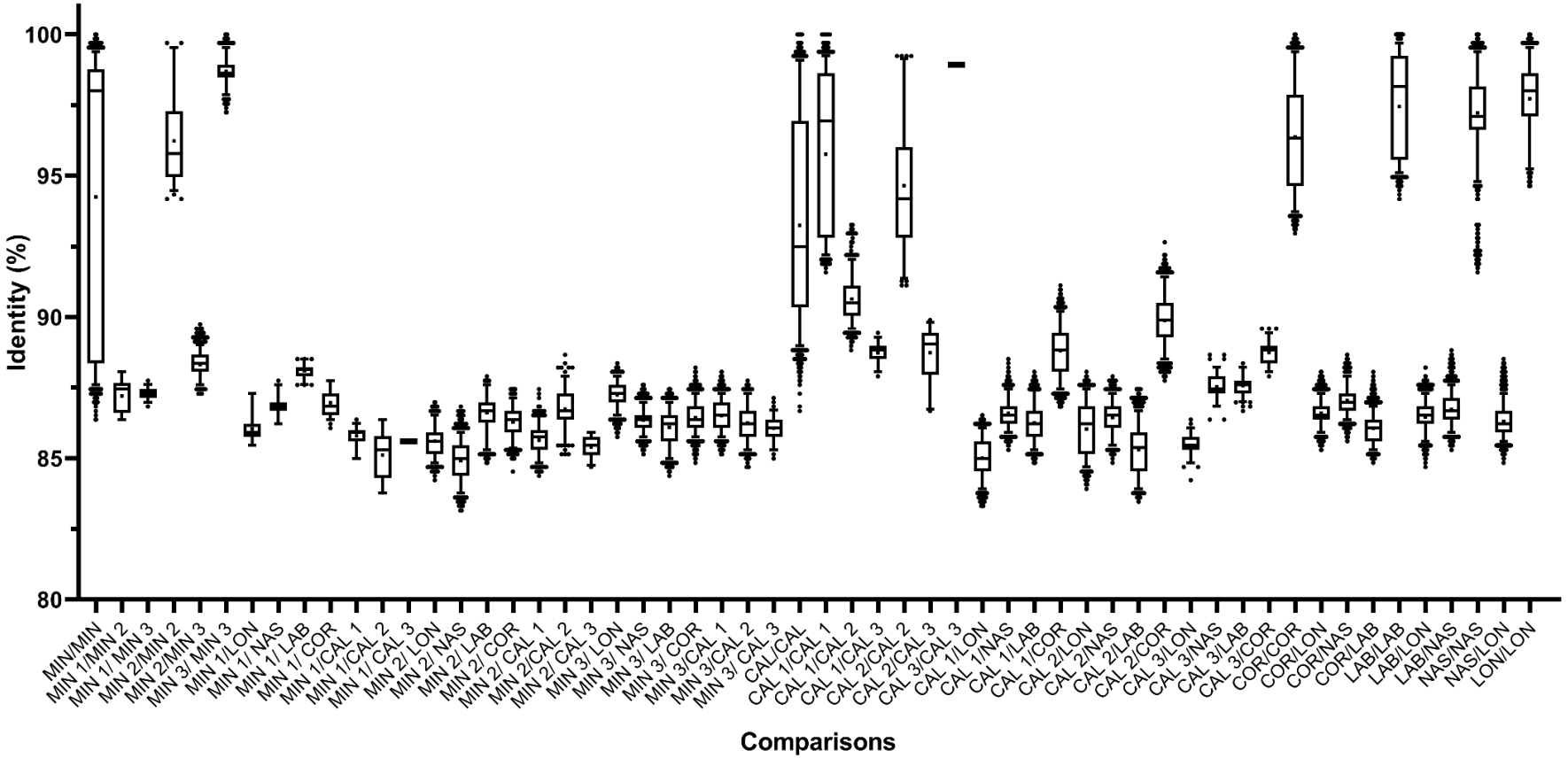
The identities between sequences within the four-remaining species (*Cor. coronatus, Cys. longibursatus, Cor. labiatus, Cyc. nassatus*) ranged at the ITS-2 locus from 98.11 to 100% and at the COI locus from 91.58 to 100%.

Figure 15 – Comparison of sequence identity between different species and operational taxonomical units (OTU) on the ITS-2 sequences.



Legend - Identities were calculated using dist.dna function and plotted as boxplots (medians and 25%/75% percentiles) with whiskers showing the 95% percentiles and outliers shown by dots. Abbreviations on the x-axis indicate the species *Cor. coronatus* (COR), *Cyc. nassatus* (NAS*), Cys. longibursatus* (LON), *Cor. labiatus* (LAB), *Cys. calicatus* (CAL) and *Cys. minutus* (MIN). The OTU's of *Cys, calicatus* (OTU I and II) and *Cys. minutus* (OTU I, II, III) are represented as CAL 1, CAL 2 and MIN 1, MIN 2 and MIN 3, respectively.

Figure 16 – Comparison of sequence identity between different species and operational taxonomical units (OTU) on the COI sequences.



Legend - Identities were calculated using dist.dna function and plotted as boxplots (medians and 25%/75% percentiles) with whiskers showing the 95% percentiles and outliers shown by dots. Abbreviations on the x-axis indicate the species *Cor. coronatus* (COR), *Cyc. nassatus* (NAS*), Cys. longibursatus* (LON), *Cor. labiatus* (LAB), *Cys. calicatus* (CAL) and *Cys. minutus* (MIN). The OTU's of *Cys, calicatus* (OTU I, II and III) and *Cys. minutus* (OTU I, II, III) are represented as CAL 1, CAL 2, CAL 3 and MIN 1, MIN 2 and MIN 3, respectively.

CHAPTER 6: DISCUSSION

The COI gene is one of the most popular genetic markers and commonly used as barcode region for diverse species. For this study, the chosen primer set was specifically designed for amplification and sequencing of a 650 bp section of the COI gene in parasitic nematodes (excluding filaroid taxa) (Duscher et al., 2015). This primer was able to amplify most of the samples and the sequences had the expected size (653 bp). For 12 samples, amplification of the COI with these primers failed. This might be due to differences in the sequence of the primer binding region, but also low DNA quality or quantity must be considered as reasons. The mitochondrial genome of nematodes is known to have a high nucleotide substitution rate, which can lead to mutations on some more conserved regions of the COI gene such as the primer binding regions (Blouin, 2002). This hypothesis is supported by observing the mutation rate on the present phylogenetic analysis, which is much higher for the mitochondrial gene when comparing with the nuclear ITS-2 sequence. Furthermore, the COI gene was able to differentiate all cyathostomin species tested in the present study, showing its usefulness as a barcode region. However, due to the absence of recombination and only maternal heritage, mitochondrial marker sequences alone can give misleading results due to lineage sorting, since different mitochondrial genotypes can co-exist and do not recombine within the same species.

The ITS-2 gene is another barcoding region widely used in many animal groups. Although amplification of the ITS-2 was more robust than that of the COI gene and was successful for all samples, it also was associated with analytical problems since PCR products sizes do vary extremely leading to difficulties in proper sequence alignment (Floyd et al., 2002; De Ley et al., 2005). This variable sequence size can be observed in the present analysis, both within some of the species or between different species. The presence of two sequences with different size within some *Cor. coronatus* samples reveals the existence of polymorphism which complicates the use of this marker as barcoding region. This polymorphism extends to *Cys. calicatus* by the presence of bands with different sizes. The fact that ITS-2 sequences were not able to discriminate two species of cyathostomins (*Cor. coronatus* and *Cys. calicatus*), that were not even placed in the same genus, confirms previous findings that the ITS-2 is not a fully reliable diagnostic marker on the species level as revealed by data on the ruminant parasitic nematode genus *Cooperia* (Ramünke et al., 2018). By adding a more variable mitochondrial marker to the analysis such as COI, the results were enhanced due to the superior barcoding properties of this sequence (Blouin, 2002). However, even though the COI sequence-based analysis was able to clearly separate between different species, it still puts species from different genera closer together than species from the same genus. Since assigning species within the subfamily Cyathostominae to a genus is based on overall similarities in morphological characters, it was expected that species within the same genus would be genetically more similar to each other than to species of other genera. While the phylograms produced from this

molecular data are not entirely congruent with the current morphological classification (Lichtenfels et al., 2008), the results are not surprising, since there are numerous examples of the incongruence between the phylogenies based on molecular and morphological data sets (McDonnell, Love, Tait, Lichtenfels, & Matthews, 2000; Gao, Zhang, et al., 2017; Qiu et al., 2018). It is important to have in mind that the characters used for phylogenetic analysis may not be the same as the morphological characters used for the identification of equine strongylids, and until a phylogenetic analysis based on morphology is available, the current morphological classification system should be the one used for strongylid identification. It is also important not to forget that a taxonomical classification is an exercise based on current information and subject to change when new hypotheses are developed, and further investigations should prove useful for establishing the systematics of equine strongylids.

When analyzing the ITS-2 phylogram, the *Cor. coronatus* and the *Cys. calicatus* OTU II cluster, showed an obvious absence of distinct nuclear haplotypes. Additionally, the identity analysis of this gene not only demonstrates a high identity value within *Cor. coronatus*, but also between this species and *Cys. calicatus* OTU II. These findings again support the evidence that the ITS-2 gene is not a reliable genetic marker to discriminate closely related species, as discussed above. For the remaining *Cys. calicatus* sequences, the presence of a polytomy could mean one of two things: there might be a rapid speciation, meaning that multiple speciation events happened at the same time; or more likely, there is a lack of information, meaning we do not have enough data to figure out which of those lineages are most closely related to each other. This polytomy was clearly resolved by adding the COI gene to the analysis, which was also able to easily differentiate both species. With this gene, we can also observe several mitochondrial haplotypes for *Cor. coronatus*. Furthermore, *Cys. calicatus* sequences were organized in three distinct clusters which had high intra-cluster identity values, favoring the hypothesis that they might represent cryptic species in a complex. This hypothesis is also supported by the fact that all sequences from the nuclear *Cys. calicatus* OTU II came from the same set of individual specimens as those from the mitochondrial *Cys. calicatus* OTU II, indicating that they did not mix with other mitochondrial haplotypes. Hereupon, mitochondrial maker COI has shown its usefulness as a barcode region by not only being able to clearly distinguish between morphologically different species, but also by demonstrating evidence for the presence of cryptic species complexes.

In all phylograms, the separation of *Cys. minutus* samples in three clades is obvious. One of the clades (*Cys. minutus* OTU I) is constituted by only one sequence, which after several PCR and molecular cloning repetitions for both genes, was considered a valid and distinct sample, excluding a possible laboratory error. Regarding the other two clades, the samples that compose them are the same for all phylogenetic trees, without mixing between mitochondrial and nuclear phylogenetic trees suggesting that they represent cryptic species. Since samples from the same host species set are present on both clades, it can be assumed that these

groups do not show a host specificity regarding different species in the genus *Equus*. Furthermore, the identity values for each gene showed high values for comparisons within each *Cys. minutus* OTU, and much lower values when comparing these OTU with other groups of species. This result supports findings by Hung, et al. (1999) on the presence of cryptic species within *Cys. minutus*. However, two samples did seem to switch clades: MIN Z F02 and MIN Z F03. One of them appeared separated in the phylogenetic tree from the combined data set (MIN Z F03), suggesting that crossbreeding can occur between both clades. Nevertheless, being consecutive sequences, it is possible that during the PCR amplification or molecular cloning, these two samples were accidentally switched when analyzing one of the genes, since these analyses where performed with a large number of samples simultaneously. To confirm if crossbreed can occur between these two clades, a repetition of the PCR amplification and molecular cloning should be performed on both samples.

Regarding *Cyc. nassatus*, the sample NAS P F01, representing a separated cluster, was identified as *Cyc. ashworth*, since its ITS-2 sequence matched one sample of this species with 99% of identity in GenBank (accession number Y08586 (Hung et al., 1997)) and its COI sequence also matched *Cyc. ashworthi* samples from different ongoing research in the same laboratory. The identification of one sample as *Cyc. ashworthi* with high level of identity, points towards a possible mistake during the morphologic identification of this specimen. During the past century, species from the genus *Cylicocyclus* were frequently misidentified with *Cyc. nassatus* and *Cyc. ashworthi* being often considered as a single species by various authors. Lichtenfels, et al. (1997) identified key characters to distinguish between these two species, which facilitated their morphologic identification and allowed the use of molecular tools to investigate and barcode these species. Although illustrated identification keys exist for cyathostomins, morphologic identification is still a difficult task, usually reserved to specialists. When considering the difficulty and the large number of worms that needed to be identified, it may be assumed that there is a risk for a possible misidentification. Both genes used in this analysis were able to separate these two species. This differentiation was expected, since Hung et al., (1997) demonstrated that it was possible to differentiate between these two species with three nuclear ribosomal genes (ITS-1, ITS-2 and 5.8S gene).

Analyzing tree topologies within *Cyc. nassatus*, it can be observed that in all trees a separated cluster was supported by high bootstrap values. Different specimens are present in such clusters depending on the gene analyzed, which indicates we have at least two very distinct nuclear and mitochondrial haplotypes, which are mixed with each other. This suggests that there is no hint for the presence of cryptic species in the *Cyc. nassatus* dataset but that different haplotypes crossbreed freely.

In contrast to findings reported by Traversa et al. (2008), there was no obvious differentiation between geographic regions (Ukraine vs. Germany) or equine host species for *Cyc. nassatus*. This absence of differentiation extends to all the other cyathostomin species in the present

study. However, in a few of the species/cryptic species such as *Cys minutus* OTU I and *Cys. calicatus* OTU III, the number of specimens within this group was very small (one and two, respectively). This small number does of course not allow any conclusions regarding geographic distribution or host specificity.

Like *Cys. calicatus*, the analysis of *Cor. labiatus* samples also showed a polytomy in the ITS-2 tree, which, however, is more likely to be due to a lack of sufficiently discriminatory molecular data than to a rapid speciation. Again, by adding the COI gene to the analysis, it is possible to solve this polytomy, supporting the evidence that COI is a better genetic marker than ITS-2 on the intra-species level. Individually, each gene divided the sequences in three clades. However, none of the clades contained the same sequences between nuclear and mitochondrial genes. Besides, the tree resulting from the concatenation of both genes, revealed a different topology and divides the sequences in two clades. This showed that diverse nuclear and mitochondrial haplotypes are present and that they mix with each other. The identity analyses also revealed higher values and a shorter range within this species when comparing within *Cys. minutus*, *Cys. calicatus* or *Cyc. nassatus*. Thus, there is no evidence that could support the presence of a cryptic species complex within *Cor. labiatus*.

The most homogenous species was *Cys. longibursatus*. The absence of distinct mitochondrial and nuclear haplotypes suggests that it is a well-defined species, on both morphologic and genomic characters. Data also showed that *Cys. longibursatus* infect all the different host species present in this analysis. Additionally, *Cys. longibursatus* has been found to be resistant to benzimidazoles (Kuzmina & Kharchenko, 2008) and was shown to have a shorter egg reappearance periods after moxidectin administration (Bellaw et al., 2018). Thus, it is easy to understand the reasons for this species being one of the most prevalent cyathostomin species found worldwide nowadays (Gawor, 1995; Silva, Costa, Santos, & Carvalho, 1999; Osterman Lind, Eysker, Nilsson, Uggla, & Höglund, 2003; Kuzmina et al., 2005; Lyons, Tolliver, & Collins, 2007).

In the animal kingdom, nuclear genes usually have slower mutation rates than mitochondrial genes, which means that when an ancestral species diverges into two or more species (speciation), some parts of nuclear genome could still be very similar between them. On the contrary, the mitochondrial genes have a faster mutation rate, meaning that we can detect speciation earlier (Eo & DeWoody, 2010). If only one of the genes would have been used in the present analysis, different conclusion would have been drawn according to the gene chosen. The nuclear gene ITS-2 was easily amplified from all samples but was shown to have poor barcoding properties. On the other hand, the mitochondrial gene COI proved to be more difficult to amplify from all samples but easily identified all species and new subspecies. By using both genes in the present analysis, we were able to complete and draw more information on the evolution of each species. This shows that using larger data sets, with more genes or even the complete worm genome, would help to solve more phylogenetic problems.

CHAPTER 7: CONCLUSION AND FUTURE PERSPECTIVES

The research presented in this thesis tried to answer specific questions about the taxonomy and phylogenetic relationships of six species of cyathostomins. The study falsified the current taxonomical classification for some genera on both analyzed genes. It is also noteworthy that there was no obvious differentiation for equid species and geographic regions (Ukaine vs. Germany).

The chosen primer sets revealed different advantages and problems. For the nuclear ITS-2 region, PCRs worked well for all samples while the primer set for the mitochondrial COI gene was unable to amplify a smaller number of sequences. Besides, ITS-2 sequences revealed the presence of polymorphism on *Cor. coronatus* and *Cys. calicatus* and demonstrated a high genetic similarity between these two species, even though morphology-based taxonomy places them in different genera. On the other hand, COI was able to clearly separate all species and addition of this gene to the analysis helped to reveal the presence of cryptic species complexes in *Cys. minutus* and *Cys. calicatus*.

In addition, it was possible to detect the presence of diverse nuclear and mitochondrial haplotypes throughout all cyathostomin species analyzed and analyze their relationship. *Cys. longibursatus* appeared to be the most homogenous species, while *Cor. labiatus* and *Cyc. nassatus* presented several mixed nuclear and mitochondrial haplotypes. Therefore, combined analysis of nuclear and mitochondrial haplotypes from the same morphologically identified specimens improved resolution of analyses and should be applied to more species and specimens from various geographic regions.

The results of this research did not only increase the existing knowledge on the molecular characteristics of cyathostomins, but also left several issues to be studied in the future. Additional analysis on some samples will help to clarify some of the problems detected by excluding possible human errors during laboratory procedures. Further investigations regarding the species included in this study could show if the subspecies identified here have subtle morphologic features, which would allow them to be distinguished and described morphologically. Additionally, larger data sets will allow to examine geographic distribution and potential host preference of species and cryptic species.

In future, it would be interesting and useful to improve the COI PCR to increase the number of samples with successful amplification, to analyze a larger number of different cyathostomin species collected from different host species and from more geographic regions, since only large data sets can help to understand the molecular epidemiology of Cyathostominae. This will in the future help to corroborate or exclude presence of cryptic species complexes and, in the long term, to study the evolution of genes responsible for evolution of anthelmintic resistance such as occurrence of benzimidazole resistance associated single nucleotide polymorphisms on a species level in the context of multi-species mixed infections.

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Research paper

Nuclear and mitochondrial marker sequences reveal close relationship between *Coronocyclus coronatus* and a potential *Cylicostephanus calicatus* cryptic species complex

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ABSTRACT

The Cyathostominae (Nematoda, Strongyloidea) parasitising equines represent a diverse group currently including 50 species. However, their taxonomy has been repeatedly revised and occasionally the presence of cryptic genospecies was suggested. Moreover, molecular- and morphology-based phylogenetic analyses give divergent results. For instance, molecular data have suggested close relationship between *Coronocyclus coronatus* and *Cylicostephanus calicatus*, although morphology-based taxonomy places them in different genera. Here, nuclear (internal transcribed spacer 2, ITS-2) and mitochondrial (cytochrome oxidase I, COI) sequences were obtained from the same individual, morphologically identified worms. In both morphospecies, two ITS-2 sequences types were observed: In *Cor. coronatus*, a small PCR product of 278 bp (nuclear haplotype group nHGBco) was always present but often in combination with a larger 369–370 bp fragment (nHGAco). In *Cys. calicatus*, either a large 370 bp product (nHGAca) or a short 281 bp amplicon (nHGBca) were found, but never both. Sequence identity between morphospecies was up to 100%. The smaller differed from the larger fragments by deletion of the region 110–198 in *Cor. coronatus* and 112–203 in *Cys. calicatus*. In COI, three and five mitochondrial haplotype groups (HGs), mtHG1co-mtHG3co and mtHG1ca-mtHG5ca were identified for *Cor. coronatus* and *Cys. calicatus*, respectively. In *Cor. coronatus*, there was no particular association of mtHG with nuclear genotypes (only nHGBco vs. both nHGBco plus nHGAco). In *Cys. calicatus* the nHGAca was always associated with the mtHG1ca, mtHG2ca or mtHG5ca whereas nHGBca was exclusively associated with mtHG3ca or mtHG4ca. Despite up to 100% identity in the nHGs, no mixing of mtHGs was observed between both species. Clear separation of certain nHGs with particular mtHGs in *Cys. calicatus*, despite the fact that the same host individuals were infected with both groups simultaneously, suggests presence of two non-interbreeding genospecies within *Cys. calicatus*, which needs further confirmation using additional samples from diverse geographical origins.

1. Introduction

Cyathostomins (Nematoda, Strongyloidea) are considered to be the most important equine parasites, due to their global occurrence, their widespread and emerging resistance against all available classes of anthelmintics, and due to the clinical disease named larval cyathostominosis, which affects horses of all age groups (Matthews, 2008). Acute clinical cases might be fatal despite comprehensive treatment; chronic

Abbreviations: ca, of *Cys. calicatus*; co, of *Cor. coronatus*; COI, cytochrome oxidase I; *Cor.*, *Coronocyclus*; *Cys.*, *Cylicostephanus*; HG, haplotype group; IGS, intergenic spacer; ITS-2, internal transcribed spacer 2; mtHG, mitochondrial haplotype group; nHG, nuclear haplotype group; nHGAca, nuclear haplotype group A of *Cys. calicatus*; nHGBca, nuclear haplotype group B of *Cys. calicatus*; nHGAco, nuclear haplotype group A of *Cor. coronatus*; nHGBco, nuclear haplotype group B of *Cor. coronatus*; OTU, operational taxonomic unit; SH-aLRT, Shimodaira–Hasegawa approximate likelihood ratio test

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cases are characterised by intermittent diarrhoea and wasting (Corning, 2009; Love et al., 1999). These parasites have a direct life cycle with adults residing in the cecum and colon of the host, where they produce eggs, which are shed with faeces. On pasture, larvae hatch and moult twice to third stage larvae which infect horses after ingestion by the host. After oral uptake, larvae penetrate the intestinal wall and reside as histotropic stages where they develop to fourth stage larvae during the up to 14 weeks. However, this development can be inhibited for example if the larvae have previously been exposed to cold temperatures. This phase also named hypobiosis can last for at least 2.5 years (Gibson, 1953). Upon an unknown stimulus, simultaneous emergence of inhibited larvae from the mucosa into the intestinal lumen is triggered, which causes inflammation and the clinical presentation of larval cyathostominosis (Love et al., 1999).

Currently, 50 species of the subfamily Cyathostominae infecting all equid species, including donkeys and zebras are recognised (Lichtenfels et al., 2008). Globally, only few experts can reliably identify these, only up to 22 mm sized parasites based on morphological characteristics, mainly of the buccal capsule (e.g. the internal and external leaf crown) and the bursa (Dvojnos and Kharchenko, 1994; Lichtenfels, 1975; Tolliver, 2000; Lichtenfels et al., 2008). Other options for diagnosis on a species level are currently very limited (Bredtmann et al., 2017).

The systematics of this extraordinary diverse group of nematode species has been revised multiple times over the years including redescription of species (Lichtenfels, 1975; Lichtenfels et al., 2002; Lichtenfels et al., 2008; Lichtenfels et al., 1998) and evidence for cryptic species in some morphospecies (Bredtmann et al., 2019; Hung et al., 1999b). Molecular and morphology-based phylogenetic approaches apparently came to distinct topologies but the morphology-based analyses were only presented at workshops and have never been published (Lichtenfels et al., 2002; Lichtenfels et al., 2008). Marker sequences that have been used in the past to diagnose cyathostomins include internal transcribed spacer 1 and 2 (ITS-1, ITS-2), intergenic spacer, cytochrome oxidase I (COI) and the 16S mitochondrial rRNA gene (Hung et al., 1999a; Hung et al., 1999b; Gasser et al., 2004; Cwiklinski et al., 2012; Hodgkinson et al., 2001; McDonnell et al., 2000; Traversa et al., 2008). In the present project, it was decided to focus on ITS-2 and COI sequences since (i) a combination of nuclear and mitochondrial markers had been shown to be advantageous in comparison to the use of only one of the genomes (Ramünke et al., 2018), (ii) the ITS-2 had been used successfully to delineate the phylogeny of cyathostomins (Hung et al., 1999a) and (iii) both markers were previously used to identify potential cryptic species and determine intra-species variability (Hung et al., 1999b; Traversa et al., 2008). In the ITS-2 based molecular phylogenetic analysis, resolution was low and members of the genera *Cylicostephanus* Ihle, 1922 and *Coronocyclus* Hartwich, 1986 were not reliably placed into separate, statistically supported clusters (Hung et al., 1999a). However, combination with more variable COI sequences was assumed to improve the resolution of phylogenetic analyses.

In the context of a larger study aiming to obtain molecular and proteomic data for a large set of cyathostomin species, very high similarity between ITS-2 sequences of *Coronocyclus coronatus* [Looss, 1900] Hartwich, 1986 and *Cylicostephanus calicatus* [Looss, 1900] Cram, 1924 in combination with intra-species variants was observed. These species were also placed in close phylogenetic proximity by two previous molecular phylogenetic analyses (Hung et al., 1999a; McDonnell et al., 2000). Therefore, the present study aimed to characterise the molecular diversity and relationship of *Cor. coronatus* and *Cys. calicatus* using a large set of morphologically identified specimens from two different geographic regions and different host species.

2. Materials and methods

2.1. Collection of worms

Adult worms were collected from eight German horses (*Equus ferus caballus)* during necropsy and from faeces of five species of equines kept at the Askania Nova Biosphere reserve, Ukraine, i.e. a domestic horse (*Equus caballus*), a wild Przewalski's horse (*Equus ferus przewalskii*), a donkey (*Equus asinus*), a Turkmenian kulan (*Equus hemionus kulan*) and a Burchell's zebra (*Equus quagga burchelli*) after anthelmintic treatment with the macrocyclic lactone product “Univerm” (0.2% aversectin C, PharmBioMed, Moscow, Russia) as described recently (Bredtmann et al., 2019). Adult worms were identified morphologically (Lichtenfels et al., 2008); specimens identified as *Cor. coronatus* and *Cys. calicatus* were included into this study. Details regarding numbers, host origin and sex of specimens are provided in Table S1.

2.2. DNA isolation, PCR and sequencing

After DNA extraction with the NucleoSpin® Tissue XS Kit (MACHEREY-NAGEL, Düren, Germany), PCRs targeting the ITS-2 (Gasser et al., 1993) and a partial cytochrome oxidase I (COI) (Duscher et al., 2015) fragments were conducted using a high-fidelity DNA polymerase (Phusion II, Thermofisher Scientific) as detailed in Table S2 and in Bredtmann et al. (2019). PCR products were cloned into the pSC-B-amp/kan vector (Strataclone Blunt Cloning Kit, Agilent) and one clone with insert per PCR fragment of the individual worm was sequenced by LGC Genomics (Berlin).

2.3. Phylogenetic analyses

Sequences were aligned using MAFFT with consideration of the predicted RNA secondary structure (Q-INS-I option) for ITS-2 (Katoh et al., 2017) and MUSCLE for COI (Edgar, 2004) followed by maximum likelihood phylogenetic analyses conducted using IQ-TREE (Schmidt et al., 2014) on the IQ-TREE server (http://iqtree.cibiv.univie.ac.at). The ModelFinder option of IQ-TREE (Kalyaanamoorthy et al., 2017) was set to auto-determination of the best model and models with FreeRate heterogeneity were included. Ultrafast bootstrapping (1000 bootstrapped alignments) (Hoang et al., 2017) and the Shimodaira–Hasegawa approximate likelihood ratio test (SH-aLRT) (1000 replicates) (Guindon et al., 2010) were chosen to obtain node support statistics. The corresponding command in IQ-TREE for ITS-2 sequences was: iqtree -s infile.fas -st DNA -m TESTNEW -bb 1000 -alrt 1000. For the protein coding COI sequences, separate models were fitted for codon positions 1 and 2 vs. codon position 3 and the command line was: iqtree -s COI_FcC_infile.fas -spp partition_file.txt -pre infile.fas -m TESTNEW -bb 1000 -alrt 1000. A combined ITS-2/COI tree was calculated using three partitions (ITS-2, COI codon position 1&2, COI codon position 3) and the command iqtree -s infile.fas -st DNA -spp partition_file.txt -pre infile.fas -m TESTNEW -bb 1000 -alrt 1000.

In order to calculate identities between sequences the dna.dist function with the method “raw” identities from the R package ape version 5.0 was used in R 3.4.3.

3. Results and discussion

3.1. Analysis of internal transcribed spacer 2 sequences

In total, 60 *Cor. coronatus* and 63 *Cys. calicatus* specimens were included into this study. ITS-2 amplification was successful for all specimens. For all *Cor. coronatus*, a 278 bp PCR (B) fragment was amplified and 41 of these *Cor. coronatus* specimens showed an additional PCR product of 369–370 bp (A). The sequences of these two PCR products differed by deletion of the bases in positions 112–203 (92 bp) of the A fragment. For *Cys. calicatus*, 46 specimens showed amplification of a

370 bp (A) fragment while 17 specimens showed a 281 bp (B) fragment. In this case, the deletion encompassed the positions 110–198 (89 bp). The number of *Cor. coronatus* A fragment haplotypes was 25, while 10 B fragment haplotypes were counted. For *Cys. calicatus*, 20 A fragment and 9 B fragment haplotypes were identified. The overall identity between fragments of the same morphospecies was in the range of 94.9–100% for *Cor. coronatus* and 96.0–100% for *Cys. calicatus* (Fig. 1A). However, identity of ITS-2 sequences between both morphospecies was also 94.9–100% indicating that ITS-2 sequences are not suitable to discriminate between *Cor. coronatus* and *Cys. calicatus*. Identity of the smaller B fragments and the homologous regions of the larger A fragments (excluding the indel region) was 95.0–100% even between the morphospecies *Cor. coronatus* and *Cys. calicatus*. When focusing only on the insertions of 92 or 89 bp that were present only in the larger A fragment, the between-species comparison revealed only 84.3–94.6% identity. In a maximum likelihood phylogenetic analysis based on ITS-2 sequences, the A and the B fragments formed separate clusters and these were used to root one cluster with the other (Fig. 2 and Fig. S1). In the cluster containing the smaller B fragments, there was virtually no substructuring observable and the *Cor. coronatus* and *Cys. calicatus* sequences were not separated from each other nor were the *Cor. coronatus* sequences from specimens with double bands (A plus B) separated from the sequences from specimens showing only the B amplicon (Fig. 2 and Fig. S1). Thus, for each species only one B type nuclear haplotype group (nHGBco and nHGBca) was identified. In contrast, for the A fragments distinct subclusters with moderate statistical support were defined as major nHGs. One cluster contained 33 *Cor. coronatus* nHG A sequences (nHGAco). The majority of *Cys. calicatus* sequences was clustered in two groups containing 36 (nHGAca1) and 8 (nHGAca2) sequences, respectively. A few *Cor. coronatus* and *Cys. calicatus* sequences were not included in these nHGs but positioned with low statistical support in the A fragment subtree (Fig. 2). There was no obvious differentiation between geographic regions (Ukraine vs. Germany) or equine host species. The fact that the ITS-2 sequences were not able to discriminate two species of cyathostomins that were not even placed in the same genus (Lichtenfels et al., 2008) confirms previous findings that the ITS-2 is not a reliable diagnostic marker on the species level as revealed by data on the ruminant parasitic nematode genus *Cooperia* (Ramünke et al., 2018).

3.2. Cytochrome oxidase I sequence analysis

Due to the missing resolution obtained by analysis of the ITS-2 sequences, a more variable mitochondrial marker with superior barcoding properties was included in the analysis (Blouin, 2002; McDonnell et al., 2000). Amplification and sequencing of a 653 bp COI fragment was achieved for 59 specimens of *Cor. coronatus* (56 different haplotypes) and 53 of *Cys. calicatus* (49 haplotypes). In contrast to the ITS-2 data, the COI-based phylogram was able to clearly separate *Cor. coronatus* and *Cys. calicatus*. For *Cor. coronatus*, the analysis further identified two major mitochondrial HGs, mtHG1co and mtHG2co, with 23 and 35 sequences, respectively (Fig. 3 and Fig. S2). A single *Cor. coronatus* COI sequence was not assigned to a cluster and was considered to belong to an additional mtHG3co. In *Cys. calicatus*, 5 mtHGs were identified with 14 mtHG1ca, 23 mtHG2ca, 9 mtHG3ca, 5 mtHG4ca and 2 mtHG5ca sequences (Fig. 3 and Fig. S2).

These mtHGs were then mapped back to the ITS-2 tree (Fig. 2). The two major *Cor. coronatus* mtHG1co and mtHG2co were both found in specimens showing only the nHGBco or both, nHGBco plus nHGAco. This shows obviously free interbreeding between the two nHGs and the two major mtHGs. Apparently, ITS-2 variants of different length are present in the *Cor. coronatus* populations. Since rRNA genes are usually present in more than one cluster in the genome, such variation in ITS length can occur in a single genome as previously described e.g. in *Ancylostoma duodenale* (Demeler et al., 2013).

In contrast to *Cor. coronatus*, the five *Cys. calicatus* mtHGs were

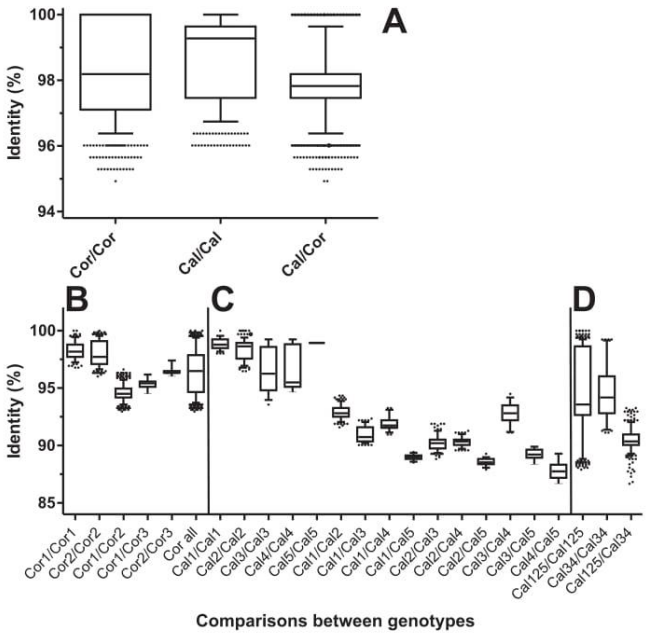

Fig. 1. Comparison of sequence identity between different species on the internal transcribed spacer 2 (ITS-2) sequence (A) or between different cytochrome oxidase I (COI) mitochondrial genotypes of the same species (B-D). Identities were calculated using dist.dna function and plotted as boxplots (medians and 25%/75% percentiles) with whiskers showing the 95% percentiles and outliers shown by dots. Abbreviations on the x-axis indicate the species *Coronocyclus coronatus* (Cor) and *Cylicostephanus calicatus* (Cal) in (A) or the mitochondrial haplotype groups Cor1-Cor3 (mtHG1co-mtHG3co) for *Cor. coronatus* and Cal1-Cal5 (mtHG1ca-mtHG5ca) for *Cys. calicatus* in (B, C). In (B), "Cor all" represents all possible comparisons between any *Cor. coronatus* sequence obtained in this study. In (D), *Cys. calicatus* mitochondrial genotypes were grouped into potential genospecies with the mtHG1ca, mtHG2ca and mtHG5ca (Cor125) being associated with the nuclear haplotype groups nHGAca, while mtHG3 and mtHG4(Cor34) were associated with nHGBca.

distributed very unevenly among the two different nHGs. While all specimens with the nuclear nHGA1ca or nHGA2ca belonged to the mtHG1ca, mtHG2ca or mtHG5ca, all specimens with the nHGBca were associated with the mtHG3ca and mtHG4ca. This strict association of certain nuclear and mitochondrial HGs suggests that there is no or little gene flow between these groups. Since the parasites came from the same host individuals, i.e. both *Cys. calicatus* genospecies were found to co-infect the German and Ukrainian horses, as well as the kulan, while in specimens from the Przewalski's horse, donkey and zebra only the nHGAca was found, it is reasonable to assume that the different *Cys. calicatus* genospecies do not interbreed and might represent different cryptic parasite genospecies.

Although the mitochondrial COI sequences provide a much better resolution in comparison to ITS-2 sequences, they alone are obviously not sufficient to correctly delineate species boundaries. In terms of raw sequence identity (Fig. 1B-D) as well as phylogenetic position (Fig. 3 and Fig. S2), the different mtHGs within *Cys. calicatus* show a degree of dissimilarity that would be comparable with a status as discrete species. In particular, distance between mtHG5ca on one and mtHG1ca/mtHG2ca on the other hand is larger than the distance between mtHG1ca/mtHG2ca and mtHG3ca/mtHG4ca.

3.3. Combined analysis of cytochrome oxidase and internal transcribed spacer 2 sequences

Finally, a combined analysis of nuclear and mitochondrial genotypes was performed. The complex PCR fragment pattern in *Cor. coronatus* (one vs. two products) cannot be explained by hybridisation with

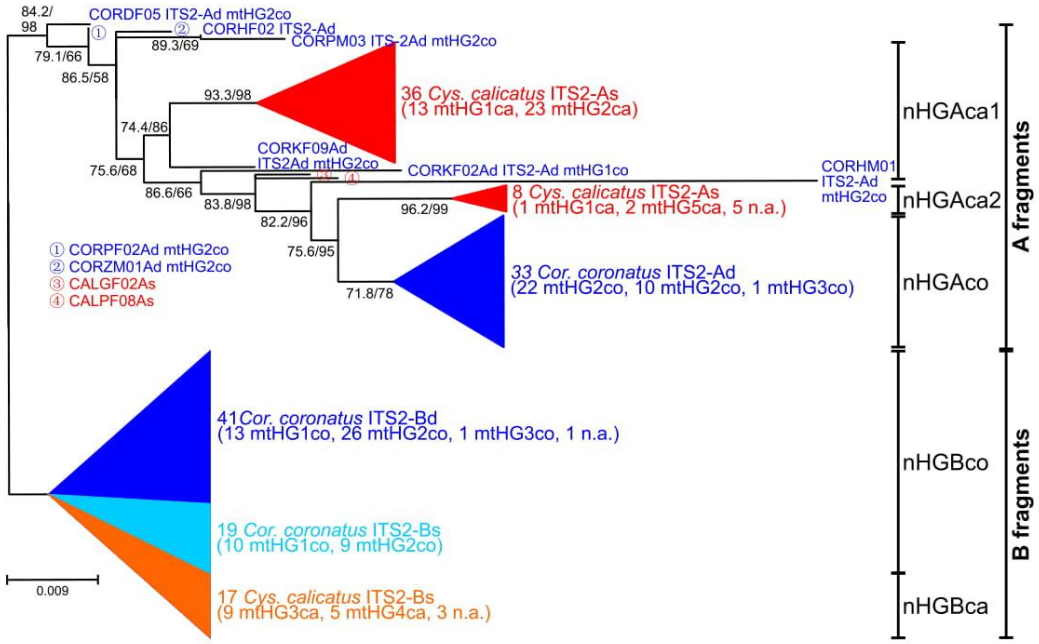

Fig. 2. A maximum likelihood phylogenetic tree was calculated using aligned internal transcribed spacer 2 (ITS-2) sequences. The scale bar represents 0.009 substitutions per site and node support was obtained by the Shimodaira-Hasegawa likelihood ratio test before and ultrafast bootstrapping (1000 replicates) after the slash. Numbers before the species names indicate the number of specimens in the group. The ITS-2 types ITS2A and ITS2B are indicated behind the species name or the specimen designation and are followed by a letter indicating whether single (s) or double (d) ITS-2 fragments were amplified. In addition to the nuclear haplotype groups (nHG), the number of cytochrome oxidase I (COI) mitochondrial haplotype groups for each nHG is provided (mtHG1co-mtHG3co for *Coronocyclus coronatus* and mtHG1ca–mtHG5ca for *Cylicostephanus calicatus*). The "n.a." indicates no successful amplification of the COI fragment from some samples. Individual specimens that were not assigned to one of the major HGs are designated according to the following code: COR/CAL for the species *Cor. coronatus* and *Cys. calicatus*; G, H, P, K, Z for the hosts German horse, Ukrainian horse, Przewalski's horse, kulan and zebra, respectively; F/M for female or male; a number indicating the individual specimen. If no mtHG is provided for individual specimens, this information was not available. The large ITS-2 A fragment (369–370 bp) and the small B fragments (278 for *Cor. coronatus* and 381 bp for *Cys. calicatus*), but not the sequences derived from each species, form distinct subtrees. In *Cys. calicatus* showing only the ITS-2 version B, only the mtHG3ca and mtHG4ca were found while *Cys. calicatus* for which only the ITS-2 A variant was amplified, only mtHG1ca, mtHG2ca and mtHG5ca were detected.

Cys. calicatus since no nHGAca was found in any *Cor. coronatus*, as identified by morphology and COI sequence. Therefore, a combined analysis of ITS-2 and COI data was conducted for all specimens for which both sequences were available. For *Cor. coronatus* ITS-2 sequences, only the B fragment was included since this was present in all specimens. The combined tree in Fig. 4 (and Fig. S3) obtained in this analysis was able to clearly separate *Cor. coronatus* from *Cys. calicatus* and in addition also clearly separated the two nHGs of *Cys. calicatus* into two groups, which was not the case using the COI sequences alone in Fig. 3. The specimens with the larger ITS-2 A bands were now assigned to an operational taxonomic unit (OTU) A and those with the smaller band to OTU B. Although these data do suggest that both OTUs represent closely related but independent species, additional marker sequences need to be included since OTU B is paraphyletic regarding OTU A. Additional markers might include the previously used IGS and 16S rRNA sequences that both have been proven to contain informative sequence variations (Cwiklinski et al., 2012; McDonnell et al., 2000). However, both markers are closely physically linked to the ITS-2 and COI sequences used here. While physical linkage cannot be avoided regarding the analysis of mitochondrial markers, combination of unlinked nuclear markers would be presumably more informative than combination of linked nuclear markers. Unlinked nuclear markers could provide evidence for isolation between genotypes, which is difficult to prove using markers with close physical proximity. Using the latter, it is

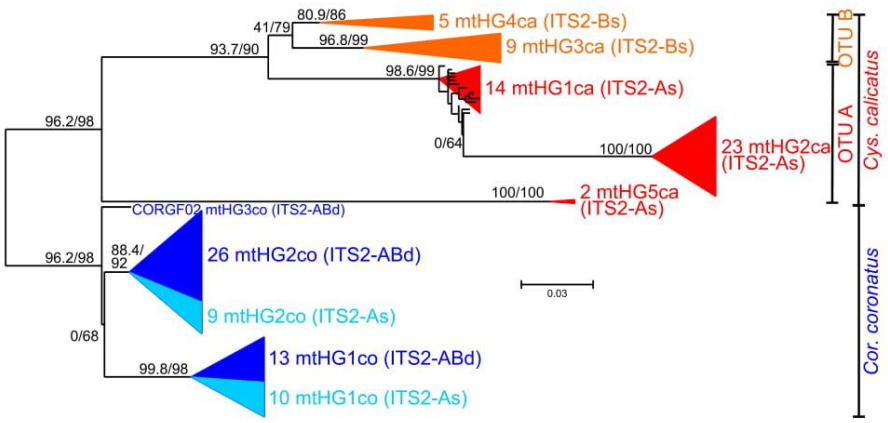

Fig. 3. Representation of a maximum likelihood phylogenetic tree calculated from the cytochrome oxidase I (COI) sequences. The scale bar indicates a distance of 0.03 substitutions per site and node support values before and behind the slash were obtained by the Shimodaira-Hasegawa likelihood ratio test and ultrafast bootstrapping. In addition to the major mitochondrial haplotype groups mtHG1co-mtHG3co for *Coronocyclus coronatus* and mtHG1ca-mtHG5ca for *Cylicostephanus calicatus*, the number of different nuclear haplotypes/genotypes in the individual clusters is provided in brackets. For *Cor. coronatus*, ITS2s and ITS2d stand for specimens from which one (ITS2B) or two (ITS2A plus ITS2B) ITS-2 variants were amplified. In the case of *Cys. calicatus*, the HGs ITS2A and ITS2B are indicated.

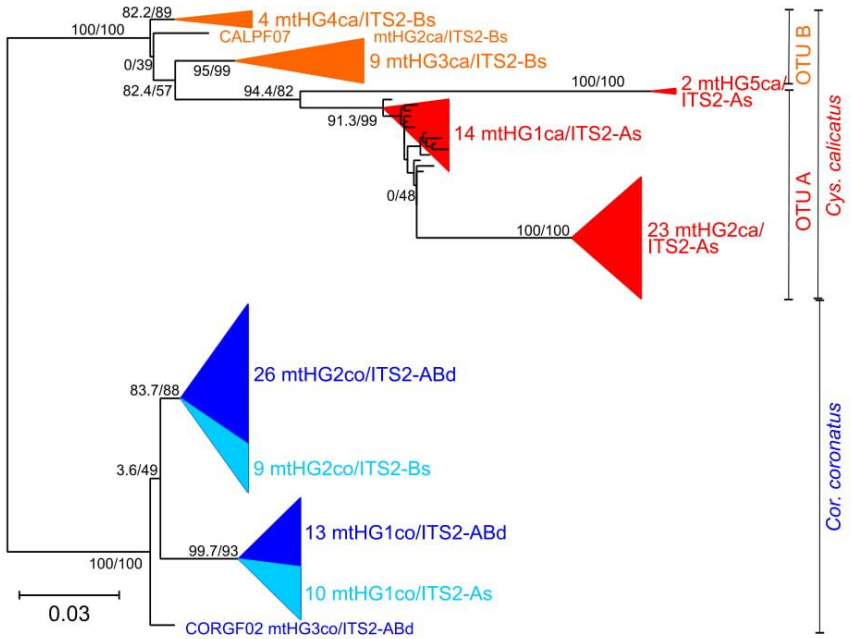

Fig. 4. Combined maximum likelihood phylogenetic tree based on cytochrome oxidase I (COI) and internal transcribed spacer 2 (ITS-2) sequences. Genotypes are designated according to mitochondrial haplotype groups (mtHGs) and presence of large (ITS2As), small (ITS2Bs) or both (ITS2ABd) are indicated. The scale bar represents 0.03 substitutions per site. Node support in terms of the results of the Shimodaira-Hasegawa likelihood ratio test and ultrafast bootstrapping are provided before and after the slash, respectively. The number of sequences in each genotype is given before the designation. Individual sequences are labelled with the specimen designation as described for Fig. 2.

very difficult to demonstrate recombination between markers.

Instead of using several independent genetic regions, the present study focused on investigating more specimens from different geographical origins. Both approaches will be required to confirm the presence of cryptic species. This approach was chosen since the initial aim was the combined analysis of molecular and proteomic markers and sample processing was optimised for this approach as described recently (Bredtmann et al., 2019). Obtaining enough protein and DNA for amplification of multiple markers was not possible so far. Unfortunately, proteomic analysis then turned out to be impossible using most of the samples included in the present study since it depends on specimens collected freshly during necropsies (Bredtmann et al., 2019) while the majority of samples included here were obtained from faeces post treatment.

3.4. Conclusions

Regarding the overall aim to obtain a reliable list of valid species of Cyathostominae and even Strongylidae infecting equines as well as a phylogenetic tree representing the most likely evolutionary history, the present study is of course only a small piece in a large puzzle. To obtain a final picture, more genetic loci, sequences of specimen from more geographic regions and more samples from equines other than domestic horses will be required. The ongoing project on molecular and proteomic characterisation of specimens identified morphologically by a recognised expert aims to contribute to this longterm goal. Additional approaches such as meta-barcoding as recently developed for ruminant gastrointestinal nematodes will presumably contribute an additional aspect to further characterise the epidemiology of this group of highly variable parasites.

The data set presented here shows high genetic similarity of *Cor. coronatus* and *Cys. calicatus* despite the fact that morphology-based taxonomy places them in different genera. Neither nuclear ITS-2 nor mitochondrial COI sequences alone were able to identify genospecies correctly while combined analysis provided a better resolution. The data of both markers together clearly separated *Cor. coronatus* from *Cys. calicatus* but also indicated the existence of discrete genospecies in what is currently assigned to the morphospecies *Cys. calicatus*.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.103956.

Declaration of Competing Interest

None.

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ANNEX 2:

Due the huge size of the trees, it is impossible to display them correctly in a paper format. To allow any reader the chance to analyze the complete trees, they are displayed here in the NEXUS format. To read the following codes, there are several software such as, for example, Fig Tree (http://tree.bio.ed.ac.uk/software/figtree/) or online platforms like Icy Tree (https://icytree.org/). Both examples are simple and free to use. Just choose the tree you wish to visualize. Copy the complete code between the horizontal lines to a text file and upload the file to the program. Or if the program allows it, copy the code directly to it.

The tree should appear rooted on the *Cys. minutus* with Shimodaira-Hasegawa and bootstrap values (SH/boot) quoted on the branches.

ITS-2 TREE

#NEXUS

begin trees;

tree tree_1 = [&R]

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end;

COI TREE

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begin trees;

tree tree_1 = [&R]
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end;

CONCATENATED ITS-2 AND COI TREE

#NEXUS

begin trees;

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